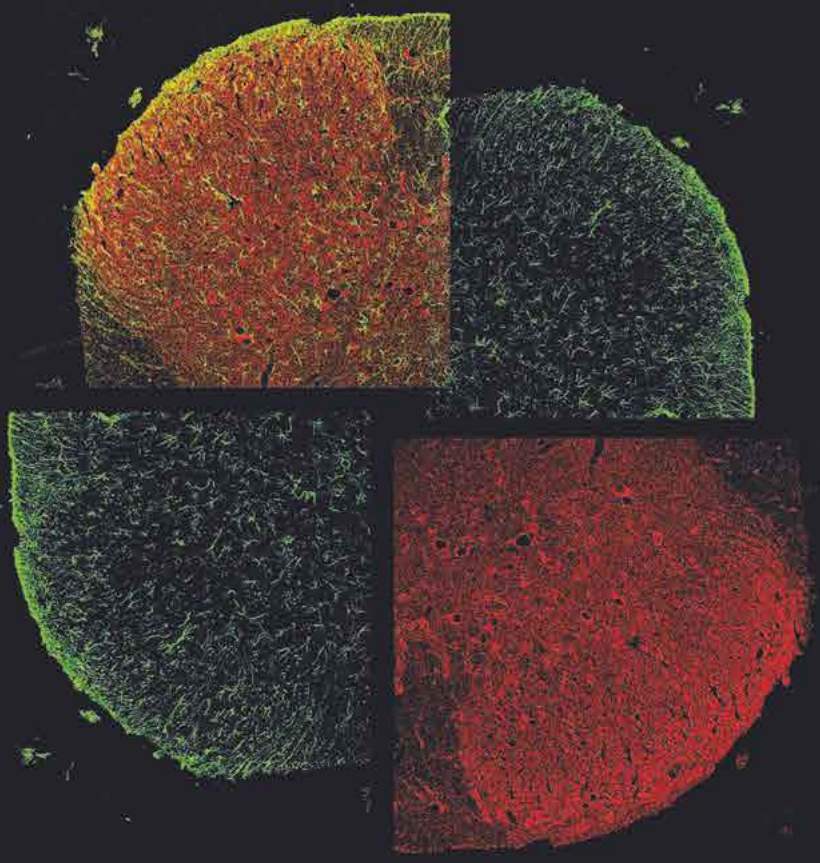
Pascal Jean-Louis Luc Vanelderen

TARGETS AND TREATMENTS FOR NEUROPATHIC PAIN

translational research from animals to humans



This research was performed and financed by the department of Anesthesiology, Pain and Palliative Medicine of the Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands and by the department of Anesthesiology, Intensive Care, Emergency Care and Pain Therapy of the Ziekenhuis Oost-Limburg, Genk, Belgium

ISBN 9789090284491

Opmaak en druk: Datawyse / Universitaire Pers Maastricht
Cover art: brain-derived neurotrophic factor and glial fibrillary acidic protein immunocytochemistry in the rat spinal cord.

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Translational research from animals to humans

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. Th.L.M. Engelen,
volgens besluit van het college van decanen
in het openbaar te verdedigen op dinsdag 11 november 2014
om 10.30 uur precies

door

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geboren op 1 april 1974
te Sint-Truiden, België

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To my loved ones: Viktor en Eva

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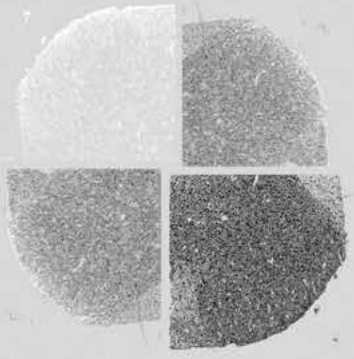

CHAPTER 1
General introduction

1.1 Definition and epidemiology of pain in general and of neuropathic pain in particular

Pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage [1]. In the clinical setting, three types of pain are distinguished: nociceptive, visceral and neuropathic pain.

Nociceptive pain arises from actual or threatening damage to non-neuronal tissues and is caused by the activation of nociceptors. It is transmitted through normal peripheral and central pain pathways and functions as a warning signal for actual or potential tissue injury thus eliciting a protective response. It tends to be of short duration, has an identifiable cause and is focal to the site of injury. Generally, nociceptive pain responds well to treatment and has a good prognosis [1].

Visceral pain originates from some but not all of the internal organs. Key features include a diffuse localization, an unreliable association with injury, referred sensations and an association with motor and autonomic reflexes. Pathways for visceral nociception are diffusely organized and include the vagus nerve, sympathetic efferent fiber pathways and the pelvic nerve. Healthy visceral tissues evoke minimal sensations. However, in the presence of inflammation silent primary afferent nerve fibers become very mechanically sensitive and highly responsive to other stimuli [2].

The third type of pain, neuropathic pain, is caused by a lesion of the somatosensory nervous system [3]. It is a chronic condition persisting beyond the point of tissue healing. Therefore, it seems to serve no biological function and, generally, has a poor response to treatment. Already in 1564, Ambroise Paré gave a detailed description of a form of neuropathic pain associated with limb amputation [4], a condition that 300 years later was termed ‘phantom limb pain’ by Silas Weir Mitchell [5]. Mitchell observed clinical signs associated with major nerve damage such as hyperalgesia (an exaggerated response to a normally painful stimulus) and allodynia (pain caused by a normally non-painful stimulus). Besides these stimulus-evoked pain sensations, spontaneous pain is also often present and described by patients as burning, throbbing or shooting [6]. At present, neuropathic pain is still defined in similar terms as those used by Paré and Mitchell: “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” [3]. Depending on where the lesion or dysfunction occurs in the somatosensory system, neuropathic pain can be peripheral or central in origin. The etiology of the nerve damage leading to neuropathic pain is diverse and may include infection, trauma, nerve compression, neurotoxins, metabolic diseases, radiation, inflammation, and tumor infiltration. A recent review of epidemiological studies concerning neuropathic pain estimates the prevalence of chronic pain with neuropathic characteristics between 6.9 and 10% in the general population [7]. Although studied for centuries, neuropathic pain still has a major impact on patients and society: neuropathic pain patients experience a poor health-related quality of life

and consume a high number of healthcare resources and costs [8, 9]. In the United States, chronic pain affects 100 million adults and costs ca. 600 billion US dollars annually. These costs exceed those of the 6 major health conditions in the US (cardiovascular diseases, neoplasms, injury and poisoning, endocrine, nutritional and metabolic diseases, digestive system diseases, and respiratory system diseases) [10]. The high prevalence of neuropathic pain in combination with a poor response to current treatments make research of neuropathic pain challenging and an absolute necessity for successful pain therapy.

1.2 Pathophysiology of neuropathic pain

In order to grasp the pathophysiology of neuropathic pain, we first need to look at the basic pain signalling pathway. This pathway consists of several elements that collaborate to transmit painful stimuli to the brain [11, 12] (Fig. 1):

1. Primary afferent nociceptors (PAN) consisting of Aδ- en C-nerve fibers, which transduce mechanical, chemical and thermal nociceptive stimuli into electrical activity and transmit this information to the dorsal horn.
2. Excitatory and inhibitory second order neurons in laminae I, II and V of the dorsal horn of the spinal cord, which integrate and modulate PAN information and transmit this information to supraspinal centers.
3. Several ascending pathways conduct this pain information to higher brain centers: the lateral spinothalamic tract (discriminative aspect of pain) and the spino-parabrachio-amygdaloid and the spino-parabrachio-hypothalamic pathways (emotional-cognitive aspects of pain).
4. In the brain, the ventroposterolateral and ventroposteromedial thalamic nuclei integrate the sensory discriminative elements of pain and transmit this nociceptive information to the primary somatosensory cortex.
5. Furthermore, the ventromedular and ventroposterioinferior thalamic nuclei transmit affective-cognitive information to the secondary somatosensory cortex, the inferior parietal cortex, the anterior cingulate cortex, the prefrontal cortex and the insular cortex.
6. Finally, cortico-limbic structures integrate pain sensation and pain affect.

Conversely, descending inhibitory pathways originating in the peri-aqueductal grey, locus coeruleus and nucleus raphe magnus reduce pain transmission in the dorsal horn of the spinal cord via the rostroventromedial medulla and the dorsolateral pontine tegmentum.

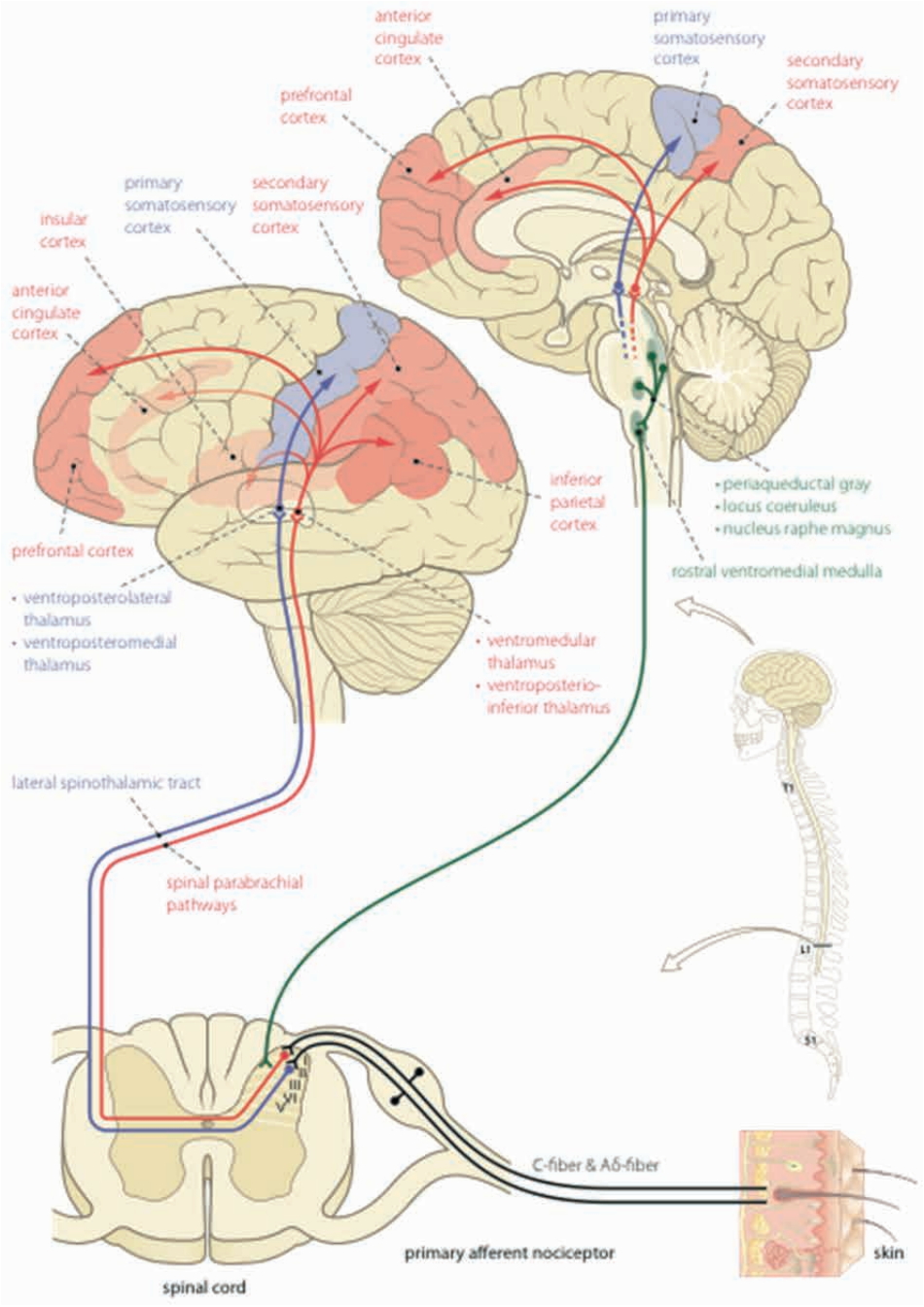

Figure 1. The pain signalling pathway. *Illustration: Rogier Trompert, Medical Art.*

Neuropathic pain ensues from sensitization within this basic pain pathway. Peripheral sensitization is caused by increased responsiveness and reduced thresholds to the stimulation of the receptive fields of nociceptive neurons in the periphery, whereas central sensitization comes from increased responsiveness to either normal or sub-threshold afferent input of central nociceptive neurons [1]. Increased neuronal responsiveness and reduced stimulation thresholds result from increased ligand-receptor interactions, from the expression of new ion channels and receptors, from the increased synaptic release of neurochemical messengers, from the activation of glial cells, and from a reduction of inhibitory signals (disinhibition). This sensitization results in facilitation of pain transmission throughout the pain signalling pathway. The different contributors to peripheral and central sensitization are summarized in Figures 2 A and B, respectively.

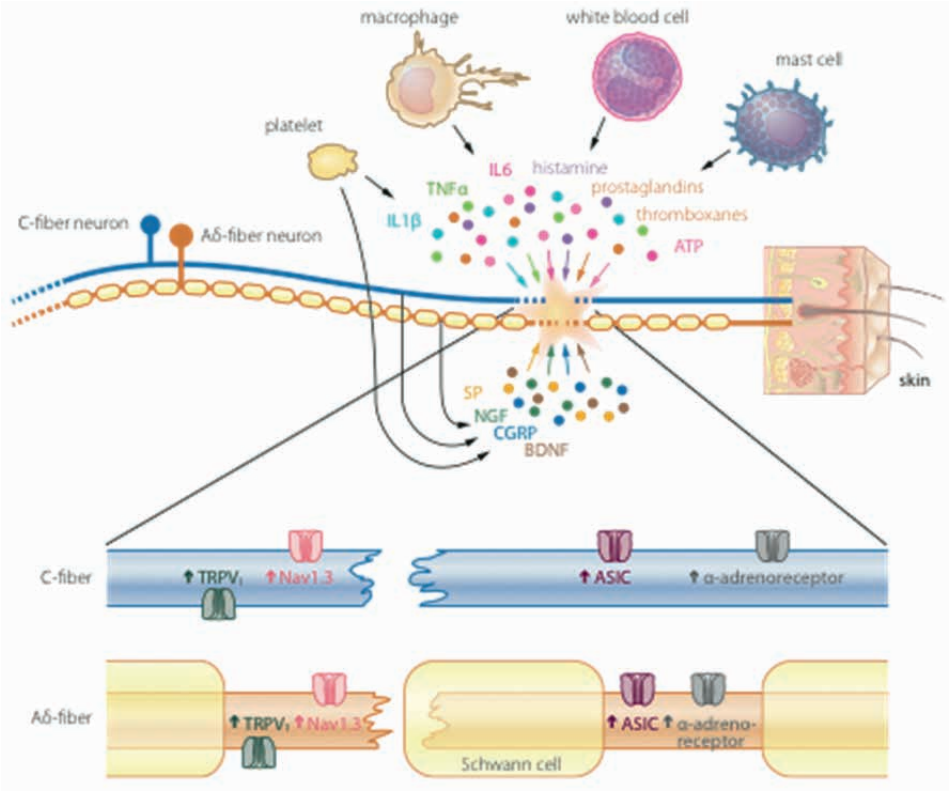

Figure 2, panel A. Peripheral sensitization. *Illustration: Rogier Trompert, Medical Art.*

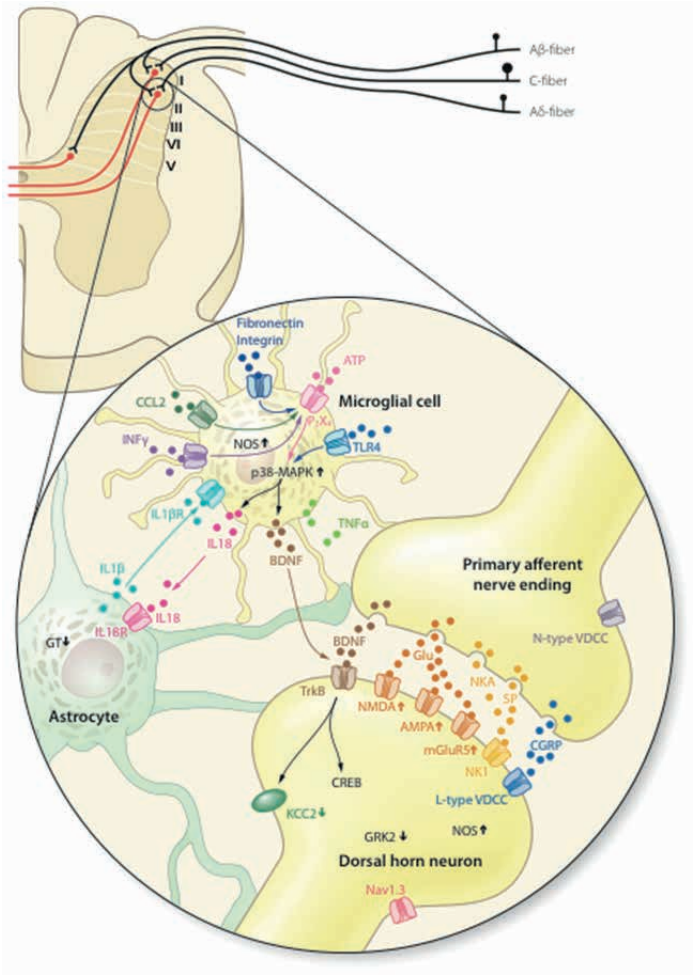

Figure 2, panel B. Central sensitization. *Illustration: Rogier Trompert, Medical Art.*
NOS: nitric oxide synthase; TLR4: toll-like receptor 4; CCL2: CC chemokine ligand 2; GRK2: G protein-coupled receptor kinase 2; CREB: cyclic AMP response element binding protein; IL18: interleukin 18; IL1β: interleukin 1β; IL6: interleukin 6; p38-MAPK: p38 mitogen-activated protein kinase; P_2X_4: purinergic receptor; KCC2: potassium chloride exporter 2; TrkB: tyrosine kinase receptor B; mGluR5: metabotrope glutamate receptor 5; ATP: adenosine-5'-triphosphate; VDCC: voltage-dependent calcium channel; TNF-α: tumor necrosis factor α, INF-γ: interferon γ; Glu: glutamate; SP: substance P; CGRP: calcitonin gene-related peptide; NMDA: N-methyl-D-aspartate receptor; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NK1: neurokinin receptor 1; NKA: neurokinin A; Nav 1.3: voltage-dependent sodium channel 1.3; NGF: nerve growth factor; TRPV1: transient receptor potential vanilloid 1; ASIC: acid-sensing ion channel.

1.3 Scientific background of the thesis research

The aim of this thesis research is to identify new targets and biological indicators ('biomarkers') and to contribute to the development of better treatments of neuropathic pain arising from a central or from a peripheral lesion (Fig. 3).

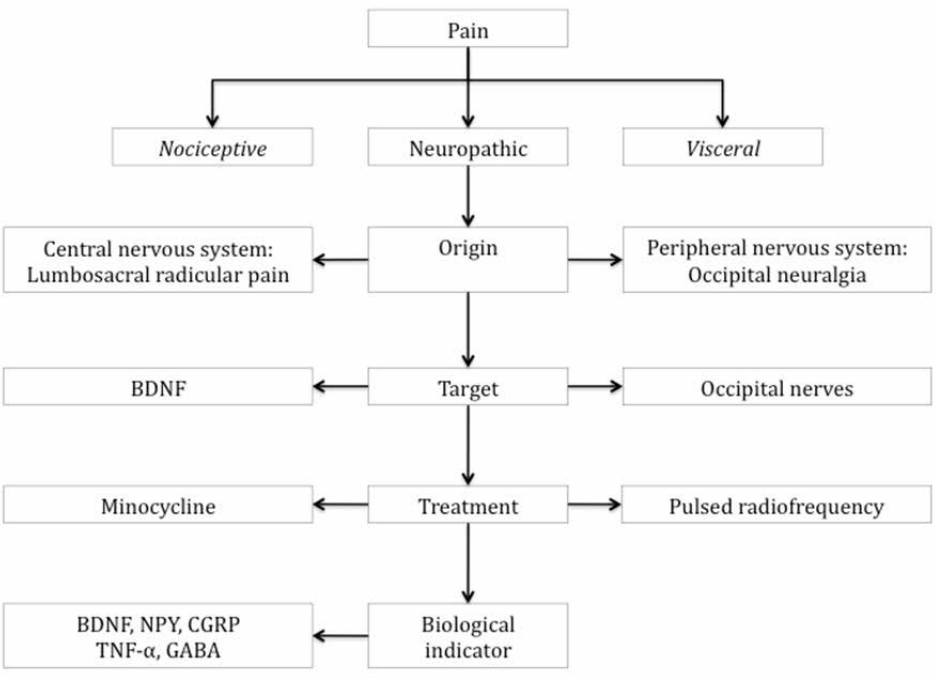

Figure 3. Schematic outline of the focus and studies described in this thesis
BDNF: brain-derived neurotrophic factor; CGRP: calcitonin gene-related peptide; NPY: neuropeptide Y; TNF-α: tumor necrosis factor α; GABA: γ-aminobutyric acid.

1.3.1 Lumbosacral radicular pain as a model for central neuropathic pain

Lumbosacral radicular pain is characterized by pain radiating into one or more lumbar and/or sacral dermatomes and may be accompanied by signs of radiculopathy (decreased motor or sensory function) or radicular irritation [13]. Lumbosacral radicular pain is much more prevalent (780-7600/100,000 population) than post-herpetic neuralgia (10-185/100,000 population), diabetic neuropathy (50-1.140/100,000 population) or complex regional pain syndrome (5-50/100,000 population) [14-16]. Nevertheless, it is among the least researched conditions with regard to neuropathic pain [9]. Furthermore, controversy exists as to the best pharmacological treatment of this condition. For these reasons, for our human research on neuropathic pain with a

central origin, we selected lumbosacral radicular pain as our model for central neuropathic pain.

1.3.2 Occipital neuralgia as a model of peripheral neuropathic pain

Another model we have used in our research on neuropathic pain is occipital neuralgia. This is a sudden non-throbbing, stabbing pain in the area of the greater or lesser occipital nerve. Tendinomuscular compression of the occipital nerves in the region of the occiput is the most frequent etiology [17]. The pain is located in the suboccipital region, radiates over the scalp and can be elicited by applying pressure over the course of the greater or lesser occipital nerve. Hypo- or dysesthesia may be present. These symptoms in combination with a short-term improvement after local anesthetic infiltration establish the diagnosis of occipital neuralgia [18]. These well-defined diagnostic criteria combined with the fact that the course of the greater and lesser occipital nerves has been very well-studied [19-22], make occipital neuralgia an ideal research model for neuropathic pain. With this model we will study the effect of pulsed radiofrequency current (PRF) on neuropathic pain with a peripheral origin.

1.3.3 Brain-derived neurotrophic factor as a key target for the treatment of neuropathic pain

Several neurochemical messengers have been implicated in the generation of neuropathic pain (Fig. 2). Among these, neurotrophins play key roles in generating and maintaining this type of pain [23-25]. Brain-derived neurotrophic factor (BDNF) deserves special attention because in recent years it emerged as a modulator of central and peripheral pain responses in spinal and peripheral nerve injury models. BDNF was the second neurotrophin to be discovered [26] and it belongs, together with nerve growth factor, neurotrophin 3 and neurotrophin-4/5, to the neurotrophin superfamily that supports the survival of specific populations of embryonic neurons. The neurotrophins interact with two kinds of cell surface receptor, viz. with the Trk family of high affinity tyrosine kinase receptors and with the low-affinity p75 neurotrophin receptor ($p75^{NTR}$). Whereas all neurotrophins bind the $p75^{NTR}$ receptor, nerve growth factor binds the TrkA receptor, BDNF and neurotrophin-4/5 bind the TrkB receptor and neurotrophin 3 binds the TrkC receptor. Although in adulthood neurons no longer remain dependent on these neurotrophins for their survival, neurotrophins still take part in crucial neural functions such as learning, memory and regulation of food intake [27, 28].

A number of animal models for chronic and neuropathic pain allow us to investigate different pain pathways and these neurotrophins under controlled experimental conditions. A prerequisite for a good model is that it mimics signs and symptoms associated with neuropathic pain in humans. For our laboratory studies we have

chosen the 'sciatic chronic constriction injury model' as described by Bennett and Xie [29] because it reliably induces allodynia, hyperalgesia and spontaneous pain.

Thanks to animal research, the involvement of BDNF in the generation and maintenance of neuropathic pain has become evident. This, however, does not mean that BDNF's role in this phenomenon is fully understood. In animal models of neuropathic pain, different lesions give rise to different patterns of BDNF expression at the lesion site, in the dorsal root ganglion and in the dorsal horn [25]. Moreover, pain is no longer the sole prerogative of neurons. Recently, microglia was found to initiate neuropathic pain and BDNF appears to be a crucial messenger in the crosstalk between neurons and microglia [30, 31]. Microglia, by way of IL-18, also entangles astrocytes in the late phase of neuropathic pain, a situation that might contribute to the maintenance of this pain [32]. As a consequence, inhibition of BDNF expression could represent an important novel step in the therapy of neuropathic pain. In order for BDNF to be a useful new target it is, however, necessary to ascertain that existing drugs for the treatment of neuropathic pain do not exert their function through inhibition of BDNF.

1.3.4 Minocycline as a new treatment for central neuropathic pain

Pharmacological management of lumbosacral radicular pain is derived from therapies of other neuropathic conditions such as diabetic polyneuropathy and postherpetic neuralgia. The latter treatments involve the increase of spinal serotonin and norepinephrine levels by administering tricyclic antidepressants such as amitriptyline and dual serotonin and norepinephrine reuptake inhibitors, or by reducing glutamate and substance P release by blocking the α2-δ subunit of voltage-gated calcium channels with gabapentin or pregabalin [33-35]. However, in randomized trials less than 50% of patients achieve adequate pain relief [36] and side-effects arising from these drugs often prohibit their use. Moreover, drugs effective in diabetic polyneuropathy and postherpetic neuralgia have failed to demonstrate superiority over placebo treatment of lumbosacral radicular neuropathic pain [37-39]. Consequently, there is a strong need for novel drugs and targets to improve current treatment of this type of neuropathic pain.

Minocycline attenuates mechanical allodynia and thermal hyperalgesia in rodent models for neuropathic pain when administered pre-emptively by inhibiting pain signalling in spinal microglia and dorsal horn neurons [40, 41]. Although beneficial effects of minocycline have been observed in the neurological and functional recovery of patients with stroke [42], spinal cord injury [43] and multiple sclerosis [44], the usefulness of this drug in the treatment of neuropathic pain in humans has never been investigated before.

1.3.5 Pulsed radiofrequency current as a treatment for peripheral neuropathic pain

For centuries electric current has been used to treat pain. In ancient Greece and Rome, pain due to gout was treated by placing patients in pools with electric eels until their feet went numb. The first controlled use of electricity in human pain treatment dates back to 1931 and concerns the treatment of trigeminal neuralgia [45]. To obtain more predictable lesion sizes, the application of radiofrequency electric current was advocated [46]. The initial assumption that heat and subsequent coagulation of nerve fibres would be responsible for interference with nerve conduction and analgesia [47] was rejected on the basis of the finding that no difference in outcome was seen when comparing two different temperatures (40°C and 67°C) applied to the cervical dorsal root ganglion to treat chronic cervical radicular pain [48]. Subsequently, a technique was developed in which the electrode temperature was kept below the neurodestructive 42°C by allowing a washout period of 480 ms after a 20 ms burst of radiofrequency current. PRF can be successful in the treatment of chronic radicular pain when applied at the dorsal root ganglion in well-selected patient groups [49-51]. Lesions of peripheral nerves often result in neuropathic pain in the nerves' dermatomal distribution areas. Because blocking peripheral nerves with local anesthetics provides only temporary pain relief, other treatments are necessary for long-term analgesia. PRF is advocated as a minimally destructive neuromodulatory therapy [52] ideal for use in the presence of non-malignant neuropathic pain. PRF at the very site of peripheral nerve injury has mainly been reported in case reports [53-57]. Only one retrospective study demonstrated superiority of PRF at the level of the thoracic dorsal root ganglion (DRG) over PRF at the intercostal nerve for chronic postsurgical thoracic pain [58]. However, when different spinal nerves contribute to the formation of one peripheral nerve or when approaching the DRG may be dangerous, it seems logical to target the peripheral nerve rather than to act upon multiple spinal DRG's with the risk of serious complications.

1.3.6 Biological indicators for neuropathic pain

Not only is there a void in the treatment of neuropathic pain, there are also no biological indicators available that allow for diagnosing neuropathic pain or for monitoring the effect of neuropathic pain treatment. Biological indicators are defined as 'any characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' [59]. This 'open' approach has permitted cardiologists to diagnose myocardial ischemia or infarction in at least five different ways:

1. Biochemically, by measuring cardiac enzymes
2. Electrically, by electrocardiogram
3. By ultrasound (echocardiography)
4. With radio-isotopes (myocardscintigraphy)

5. By cardiac and coronary catheterization

However, in pain medicine, physicians still rely on rather subjective questionnaires to clinically detect or differentiate between various forms of pain, in spite of the vast current knowledge of multiple biochemical pathways with their numerous signalling molecules that have been uncovered through animal research. Imaging studies such as functional magnetic resonance imaging [60, 61], positron emission tomography [62] and proton magnetic resonance spectroscopy [63] are emerging as promising new tools to detect chronic and neuropathic pain. These techniques demonstrate biochemical changes in various brain regions associated with pain processing. However, due to their time-consuming nature and expensiveness, they have not found their way into daily clinical practice. It would enhance the clinical practice if the biochemical substances detected by these techniques could be measured more easily in blood and cerebrospinal fluid. As the perception and expression of pain appears to be the result of complex processes that are influenced by emotions and cognitions that change its perception and expression, biological indicators will probably not permit us to accurately measure the degree of pain patients perceive. Nevertheless, we can search for biological indicators that qualitatively distinguish nociceptive from neuropathic pain. In addition, the use of biological indicators in humans can give us, albeit indirect, insight into the mechanisms underlying tissue damage, nerve injury and neuropathic pain because direct spinal or nerve tissue sampling as performed in animal research would lead to the neuropathic pain condition that we are trying to treat. Therefore, in this thesis research we have investigated the concentrations of key molecules known to participate in the generation and maintenance of neuropathic pain in the blood and cerebrospinal fluid of patients suffering from neuropathic pain.

1.4 Research questions

In this thesis, the following research questions have been addressed:

- What is the role of BDNF in animals suffering from neuropathic pain?
- Do amitriptyline, gabapentin and minocycline reduce neuropathic pain behavior in a rat chronic constriction injury model via BDNF?
- Can minocycline and amitriptyline reduce lumbosacral radicular pain in humans?
- What is the current state of the art concerning occipital neuralgia?
- Can PRF at the level of the occipital nerves reduce neuropathic pain in occipital neuralgia?
- Can neurotransmitters and other neurochemical messengers be used as biological indicators for neuropathic pain?

1.5 Outline of this thesis

In **Chapter 2** an overview will be given of the scientific evidence that BDNF is involved in the generation of neuropathic pain in various animal models.

In **Chapter 3** we will report on an investigation on the effects of current medical therapies on neuropathic pain behavior in rats and their possible actions on BDNF expression in the spinal dorsal horn.

Chapter 4 describes how the findings of our animal research have been used in a clinical trial, studying the effect of minocycline and amitriptyline on lumbosacral radicular pain in humans.

Chapter 5 is a review of the state of the art regarding occipital neuralgia and its treatment.

In **Chapter 6** the effects of PRF on occipital neuralgia, as a model of peripheral neuropathic pain, will be presented.

In **Chapter 7** neurochemical messengers involved in the generation and maintenance of neuropathic pain will be examined for their usefulness as biological indicators.

In **Chapter 8** we will discuss the results of this thesis research in a broad and integrated scientific context, and several suggestions for future research will be given.

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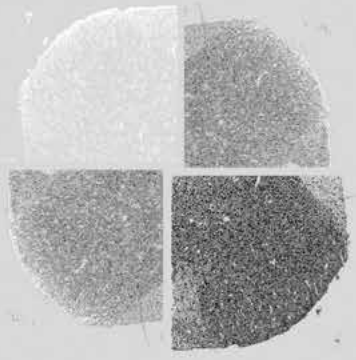

CHAPTER 2

The role of brain-derived neurotrophic factor in different animal models of neuropathic pain

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Published in European Journal of Pain, 2010 May;14(5):473.e1-9.

Abstract

Even in present day pain therapy, neuropathic pain remains a challenge for clinicians to treat and a challenge for researchers to investigate. Different animal models have been developed to mimic neuropathic pain. Neurotrophins such as nerve growth factor, brain-derived neurotrophic factor and neurotrophin 3 have been studied extensively in these models, yet few review articles concerning brain-derived neurotrophic factor have been published. This article reassesses the literature concerning brain-derived neurotrophic factor expression in the sciatic nerve chronic constriction injury model, the sciatic nerve transection model, the spinal nerve ligation model and the spinal nerve transection model and discusses differences in regulation of brain-derived neurotrophic factor between these models and their causality with neuropathic pain.

2.1 Introduction

Neuropathic pain is caused by diseases or trauma to the central or peripheral nervous system [1]. With an estimated prevalence of 1.5%, the burden of neuropathic pain on patients and healthcare systems appears to be potentially large: neuropathic pain patients experience a poor health-related quality of life and consume a high level of healthcare resources and costs [2, 3]. Devoid of specific drug therapy and poorly responsive to traditional pain medication [4] this condition still embodies a challenge to researchers and clinicians [5]. A number of animal models for chronic neuropathic pain have been established and validated: chronic constriction injury (CCI) of the sciatic nerve [6], partial sciatic nerve ligation [7], L5 and L6 spinal nerve ligation (SNL) [8], sciatic nerve transection [9], L5 spinal nerve transection [10] and spared nerve injury [11]. Neurotrophins play key roles in neuropathic pain [12, 13] and are researched extensively in these animal models. Brain-derived neurotrophic factor (BDNF) was the second neurotrophin to be uncovered in 1980 [14]. Together with nerve growth factor (NGF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5), BDNF belongs to the neurotrophin superfamily that supports the survival of specific populations of embryonic neurons. The neurotrophins interact with two categories of cell surface receptors: the Trk family of high affinity tyrosine kinase receptors and the low-affinity p75 neurotrophin receptor ($p75^{NTR}$). Whereas all neurotrophins bind the $p75^{NTR}$ receptor, NGF binds TrkA receptors, BDNF and NT-4/5 bind TrkB receptors and NT3 binds TrkC receptors. Although in adulthood neurons no longer remain dependent on these neurotrophins for their survival, neurotrophins still take part in important neuronal functions such as learning, memory [15] and food intake [16]. In recent years BDNF emerged as a modulator of central and peripheral nociceptive responses. In a general review about BDNF, Merighi [17] concluded that the role of BDNF in inflammatory pain models is well defined, although due to the multiplicity of experimental approaches, the correlation between BDNF and neuropathic pain is still unclear. The present review article concentrates on the sciatic nerve chronic constriction injury model, the sciatic nerve transection model, the spinal nerve ligation model and the spinal nerve transection model as models for neuropathic pain and categorizes the current literature about the expression of BDNF in the peripheral nerve, dorsal root ganglion and dorsal horn with emphasis on the site and type of nerve lesion used to mimic neuropathic pain. The type of neurons and intracellular transduction pathways thought to be involved in the expression of BDNF will be reviewed.

2.2 Literature Search Methods

A Medline (PUBMED) search using the terms bdnf AND sciatic AND ligation, bdnf AND sciatic AND constriction, bdnf AND sciatic AND transection, bdnf AND spinal AND nerve

AND ligation, bdnf AND spinal AND nerve AND constriction, bdnf AND spinal AND nerve AND transection was performed. Articles with a publication date up to January 1, 2009 were included. This resulted in 11, 13, 45, 20, 14 and 48 references, respectively. The literature was categorized in experiments using a sciatic nerve chronic constriction injury, a sciatic nerve transection injury, a spinal nerve ligation model and a spinal nerve transection (SNT) model. The results and figures pertaining to the spinal nerve ligation and spinal nerve transection models have been published online as results S1 and figure S1.

2.3 Results

2.3.1 BDNF expression in the sciatic nerve chronic constriction injury model

Following a loose chronic constriction injury of the sciatic nerve, Ha [18] found an increased BDNF expression in small, medium and large neurons in the L4 and L5 DRG and in axonal fibers in the medial superficial and deeper laminae of the ipsilateral L4/5 dorsal horn (DH) to where these neurons project. Also using a sciatic CCI model, Obata [19] employed activating transcription factor 3 (ATF3) expression as a neuronal injury marker. Although the same procedure was performed in all rats, the extent of damage as evidenced by the number of ATF3-labeled neurons differed significantly. Rats were divided into 3 groups according to the percentage of ATF3-immunoreactive (IR) neurons: group 1 (<12.5%), group 2 (12.5–25%), and group 3 (>25%). The investigators found that only rats with substantial nerve damage (groups 2 and 3) showed thermal hyperalgesia, whereas only the rats in group 2 developed tactile allodynia. In groups 1 and 2, BDNF-IR neurons were primarily of small or medium diameter whereas in these groups the injured neurons were mainly of medium or large diameter. In group 2, there was no co-localisation for ATF3 and BDNF in neurons indicating that the increased BDNF expression occurred mainly in non-injured neurons. In group 3 small, medium- and large-sized neurons were labeled for ATF3-IR and small, medium and large neurons were labeled for BDNF-IR, but no co-localisation experiments were made in this group. The pattern of BDNF labelling in the latter group bears a resemblance to the results of Ha [18]. Elucidation of the cause of this differential expression of BDNF is provided by the pathophysiology of CCI. With loose CCI of the sciatic nerve, at the time of tying, the ligatures just barely reduce the nerve diameter. Over time, the ligatures evoke intraneural edema, resulting in constriction of the nerve. Electron microscopic studies of the sciatic nerve 10 to 28 days after surgery revealed destruction of most Aβ- and Aδ-fibers distal to the CCI. In contrast, damage to C-fibers was variable, ranging between 30 and 80% [20]. Physiological studies also demonstrated that 90% of Aβ- and Aδ-fibers and 30% of C-fibers do not conduct through the CCI 3 days after surgery [21]. Thus in a CCI model C-fibers are relatively spared compared to the larger Aβ and

Aδ fibers. NGF is produced abundantly around degenerating Aβ and Aδ fibers by Schwann cells and macrophages [22-24]. This NGF is retrogradely transported by intact C-fibers to the dorsal root ganglion (DRG), leading to an increased production of BDNF in small to medium neurons. Corroborating the hypothesis of changed target derived NGF as cause of altered BDNF expression, Obata [25] administered recombinant NGF intrathecally (i.t.) in Sprague-Dawley rats and found an increased BDNF expression in small and medium DRG neurons.

Intracellular signal transduction pathways involved in the pathophysiological mechanisms of neuropathic pain after sciatic nerve CCI also vary according to the damage inflicted to the DRG neurons [25]. The mitogen-activated protein kinase (MAPK) pathway involvement in neurotrophin dependent survival and differentiation of developing peripheral neurons has been clearly established [26-29]. The MAPK family includes the extracellular signal-regulated protein kinases (ERK), p38 MAPK, c-Jun N-terminal kinase (JNK) and ERK5. ERK is involved in cellular growth and differentiation, whereas p38 and JNK participate in injury responses and cell death. After CCI there is a phosphorylation of ERK predominantly in injured ATF3-positive medium and large DRG neurons and in satellite glial cells. CCI also increased the expression of NPY in these neurons. Treatment with the MAPK kinase 1⁄2 inhibitor, U0126, suppressed CCI-induced mechanical allodynia and partially reversed the increase in NPY expression. In contrast, CCI induced the activation of p38, mainly in uninjured ATF3-immunonegative unmyelinated small to medium DRG neurons and in satellite glial cells. In intact DRG neurons, the p38 inhibitor, SB203580, reversed CCI-induced mechanical allodynia and heat hyperalgesia, and also blocked the increase in BDNF expression. I.t. administration of NGF increased BDNF expression in small-to-medium-sized neurons, which could be reversed by SB203580, whereas anti-NGF increased NPY in medium-sized and large-sized neurons, which could be partially blocked by U0126.

CCI not only induces changes in BDNF expression in the DRG but also in the dorsal horn (DH). Sprague-Dawley rats exhibiting thermal hyperalgesia after sciatic CCI exhibited a significant increase in the concentration of BDNF in their lumbar spinal DH [30]. Following the disappearance of thermal hyperalgesia 28 days after loose ligation of the sciatic nerve, there was no difference in DH BDNF level between control rats and animals with sciatic ligations, suggesting a close association in the timeline of the development and disappearance of behavioural signs of neuropathic pain with changes in BDNF level in the lumbar spinal DH. Furthermore, Yajima [31, 32] established an increase in BDNF and its full-length TrkB receptor in the ipsilateral superficial laminae of the DH after sciatic CCI. I.t. application of BDNF-antibody or TrkB/Fc prevented the development of thermal hyperalgesia and abolished the increase in full-length TrkB receptor in the dorsal horn. TrkB/Fc contains the extracellular ligand-binding domain of a TrkB receptor followed by the hinge and Fc region of human IgG1 and acts as TrkB receptor body to sequester endogenous BDNF and neurotrophin-4/5. The induced thermal hyperalgesia was also reversed by i.t. administration of antibody raised against

the full-length TrkB receptor, K252a (an inhibitor of the tyrosine kinase activity for the neurotrophin receptor) or the selective PKC inhibitor Ro-32-0432, but i.t. injection of an antibody to the truncated TrkB receptor or to NT4 failed to relieve thermal hyperalgesia. Additionally, a single i.t. injection of BDNF produced a marked and long lasting thermal hyperalgesia and tactile allodynia in normal mice after the injection. This effect was abolished by i.t. pre-treatment with K-252a or the selective PKC inhibitor Ro-32-0432. Balasubramanyan [33] used spinal cord slices of rats that underwent a sciatic CCI and Lu [34] used defined-medium organotypic cultures consisting of embryonic Sprague-Dawley spinal cord and DRG neurons treated with BDNF 200 ng/ml. Both investigators found identical results with patch clamp techniques, namely increases in excitatory synaptic drive to putative excitatory 'delay' firing neurons in the substantia gelatinosa but decreased synaptic drive to putative inhibitory 'tonic' firing neurons. Effects of BDNF on putative excitatory 'delay' neurons were exclusively presynaptic, whereas both pre- and post-components were involved in BDNF's action on putative inhibitory 'tonic' neurons. These BDNF-induced changes in synaptic drive contributed to an overall increase in DH excitability thus promoting a state of central hypersensitivity as seen in the sciatic CCI model. The mechanism by which BDNF enhances synaptic transmission could be phosphorylation of CREB Ser^{133} by acting on its high-affinity TrkB receptor, thus promoting a cascade of events leading to synapse specific efficacy [35].

An increasing number of studies indicate that pain is not solely mediated by neurons but that microglia and astrocytes have an active role in the initiation and maintenance of neuropathic pain. Zhang and De Koninck [36] found that spinal microglia were activated after sciatic CCI by monocyte chemoattractant protein-1 produced by damaged DRG neurons which is subsequently transported to the afferent nerve terminals in the DH. An intact dorsal root seems essential to convey DRG mediated signals for activation of spinal microglia [37]. Another way in which microglia may contribute to neuropathic pain is through the activation of their $P2X_4$ receptor by ATP [38]. This leads to an influx of extracellular calcium and subsequent activation of p38 MAPK resulting in an increased synthesis and release of BDNF through soluble N-etylmaleimide-sensitive factor attachment protein receptor dependent exocytosis [39]. Furthermore, the release of BDNF is biphasic with an initial release of a pre-existing pool of BDNF followed by an increase in *de novo* synthesis of BDNF. Coull [40, 41] discovered that BDNF released by ATP-stimulated microglia causes a depolarizing shift in the anion reversal potential of lamina I neurons by ways of a reduction in the expression of the potassium-chloride exporter KCC2. Normally inhibitory anionic synaptic currents activated by GABA are thus rendered excitatory leading to a disinhibition of the DH and neuropathic pain. In a more recent study Lu [42] corroborated the findings of their earlier study and moreover identified the inhibitory neurons as GABAergic and the excitatory neurons as non-GABAergic using glutamic acid decarboxylase. Even more important in this study was the fact that addition of activated microglia to organotypic cultures increased the overall excitability of the DH as was seen by adding

BDNF. The effect of activated microglia was abrogated by the recombinant protein TrkB-d5, which binds and inactivates all neurotrophins that interact with TrkB receptors, confirming the earlier findings that activated microglia may be an important source of BDNF in neuropathic pain syndromes. Another possible mechanism by which BDNF induces central hypersensitivity is by enhancing NMDA receptor-mediated responses in the DH [43]: the ventral root potential is routinely used as an indirect but accurate measure of spinal excitability. NMDA-evoked ventral root depolarization amplitudes increased significantly after BDNF superinfusion in isolated hemisected spinal cords.

Finally, gonadal hormones appear to influence BDNF expression and pain behavior. Zhao [44, 45] studied the effect of estrogen on neuropathic pain using a sciatic CCI model in female Sprague–Dawley rats. Animals with high estrogen levels responded significantly faster to thermal nociceptive stimuli then ovariectomized rats. The former rats also had higher BDNF mRNA in the ipsilateral DRG's and a higher BDNF protein level in the spinal cord, suggesting the presence of an estrogen-responsive element in the BDNF gene. The summarized data are found in Fig. 1.

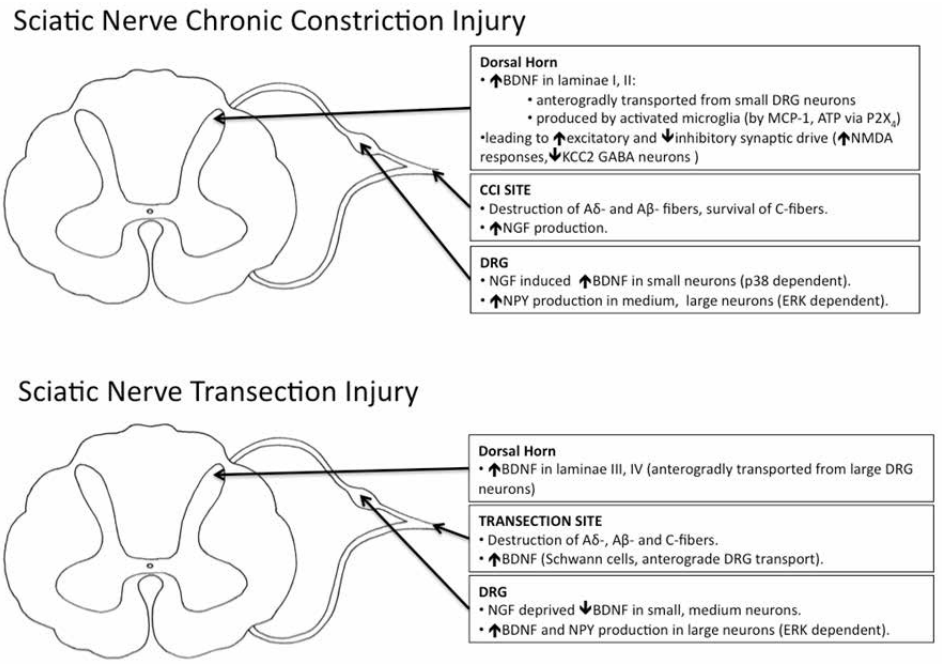

Figure 1. BDNF expression in the sciatic nerve chronic constriction injury and sciatic nerve transection models (BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor, DRG: dorsal root ganglion, NMDA: N-methyl-D-aspartate, KCC2: potassium-chloride co-transporter 2, GABA: γ-aminobutyric acid, NGF: nerve growth factor, CCI: chronic constriction injury, p38: p38 mitogen-activated protein kinase, ERK: extracellular signal-regulated protein kinases, NPY: neuropeptide Y, MCP-1: monocyte chemoattractant protein-1, ATP: adenosine triphosphate, $P2X_4$: purinoreceptor $P2X_4$, ↑: increase, ↓: decrease).

2.3.2 BDNF expression in the sciatic nerve transection model

Meyer [46] and Funakoshi [47] found a continuous slow increase in BDNF mRNA in the sciatic nerve after transection, starting 3 days after the lesion and reaching maximal levels 3-4 weeks later. The final levels were 10 times higher than those of NGF mRNA. The increase in BDNF was independent of activated macrophages and IL-1β and was probably generated by Schwann cells, findings corroborated by Friedman using myelin-deficient Trembler mice [48]. Tonra [49] also demonstrated an increased transport to the injury site of BDNF after a sciatic crush lesion. The source of this BDNF seemed to be sensory neurons in the DRG. So, in addition to an increased BDNF production at the injury site by Schwann cells, an additional amount of BDNF may be supplied through anterograde transport of BDNF produced by DRG neurons.

Cho [50] and Zhou [51] were the first to demonstrate a switch in phenotype in primary sensory neurons after sciatic nerve transection in Sprague-Dawley rats. Normally expressed in small to medium DRG neurons, BDNF was now expressed in larger sensory neurons (with a surface of >600 μm^2) in the ipsilateral L4 and L5 DRG. There was no significant change in the contralateral DRG. In the DH, BDNF-IR, which is normally localized in large electron-dense core vesicles in neurons of the superficial layers, now increased in laminae III and IV and diminished in laminae I and II. Michael [52] and Kashiba and Senba [53] confirmed these findings in a sciatic nerve transection model in male Wistar rats. They found a significant increase in BDNF expression in ipsilateral TrkB- and TrkC-containing medium-to-large DRG neurons. BDNF-IR was extensively co-expressed with galanin and NPY in large DRG neurons. BDNF expression in the DH also shifted towards the deeper lamina IV as was confirmed by Walker [54]. Since BDNF expression in small DRG neurons is NGF-dependent, deprivation of target-derived NGF serves as a basis for downregulation of BDNF expression in these neurons and their endings in laminae I and II of the DH. Until recently, large DRG neurons were believed to be independent of NGF due to a lack of TrkA receptors, but Karchewski [55] showed that 19% of DRG neurons express both TrkA and TrkC receptors and that the expression of two or three Trk receptor mRNAs by the same neuron occurs almost exclusively in medium-sized to large neurons. Therefore, in normal circumstances, NGF may inhibit BDNF expression in large DRG neurons. With sciatic transection the negative feedback loop is interrupted and BDNF expression stimulated. Corroborating this hypothesis was the finding by Obata [56] that i.t. administered anti-NGF induces an increase in BDNF and NPY expression in medium to large DRG neurons. Another possible explanation is an increase in intracellular calcium concentration, either by direct influx or through the NMDA receptor, which would upregulate BDNF through a CREB transcription factor-dependent mechanism. Finally, a negative feedback by target-derived NT3, the survival factor for large DRG neurons, could be a possibility. In view of the fact that not only target-derived NGF but also NT3 is deficient after nerve lesion, inhibition in large neurons disappears and BDNF expression is upregulated.

Supporting this evidence is a study by Ohara [57] where sciatic nerve transection induced a rise in NPY-IR in medium and large DRG neurons and in laminae III and IV of the DH, which is abolished by exogenous administered NT3.

Obata [56] looked into the intracellular signal transduction pathways involved in regulating the gene expression of BDNF in primary afferent neurons after sciatic nerve transection. Axotomy induced the activation of ERK and upregulation of BDNF mainly in medium- and large-sized DRG neurons and in satellite glial cells as did the application of anti-NGF.

Marcol [58] provided evidence for a relationship between neuropathic pain and BDNF at the site of nerve injury by supplying the proximal nerve stump of transected sciatic nerves with connective tissue chambers filled with fibrin only, with fibrin and BDNF, or with fibrin and anti-BDNF. Rats usually began to self-mutilate the denervated limbs within the first week following nerve transection. Local application of BDNF not only brought forward the onset of autotomy but also increased the incidence and severity of autotomy behaviour. In contrast, local inhibition by anti-BDNF antibody decreased neuroma formation and the incidence and severity of autotomy, whereas survival and regeneration of DRG cells were not affected. Several explanations are possible to elucidate these findings. First, peripheral BDNF stimulates neuroma formation [58]. Since neuromata are known to cause neuropathic pain, local inhibition of BDNF will prevent this effect. Secondly, sequestration of locally produced BDNF by means of BDNF-antiserum averts proximal transportation, thereby inhibiting nociceptive pathways in the DRG or DH. Thirdly, anti-BDNF is transported proximally and inhibits BDNF at the DRG or DH.

In a brachial plexus avulsion model, Quintao [59] was able to reproduce the results by Marcol [58], by local application of anti-GDNF, anti-BDNF, anti-NT3 and anti-NGF antibodies at the time of surgery. Mechanical hypernociception was prevented, with highest potency of anti-GDNF followed by anti-BDNF, anti-NT3 and anti-NGF antibody.

Neuropathic pain can be relieved not only by inhibiting BDNF at the site of sciatic nerve transection but also by inhibiting BDNF at the level of the DRG or DH. Inhibiting the intracellular transduction pathway leading to an increased BDNF production in large DRG neurons by administering U0126 suppressed the axotomy-induced autotomy behaviour [56]. Ultimately, Park [60] noticed a reduction in expression of voltage-gated potassium channels Kv 1.2, 1.4 and 4.2 in DRG neurons of adult male Sprague-Dawley rats after sciatic nerve transection. In *in vitro* cultured DRG neurons application of BDNF and NT3 but not of NGF produced similar effects on Kv 1.2 and 1.4. Kv 4.2 expression that could not be altered by any neurotrophin. The summarized data are found in Figure 1.

2.4 Discussion

In the sciatic CCI model small C-fibers are relatively spared compared to larger Aβ and Aδ fibers. Wallerian degeneration and inflammation induce NGF production in Schwann cells and macrophages and this NGF is retrogradely transported by intact C-fibers to small and medium DRG neurons. In these neurons NGF binds its TrkA receptor, increasing BDNF production by a p38 MAPK-dependent intracellular transduction pathway. BDNF is transported to the superficial laminae of the DH where in turn it binds its TrkB receptor inducing thermal hyperalgesia and mechanical allodynia through secondary pathways involving PKC. Moreover, primary sensory neurons release BDNF in an activity-dependent manner indicating that BDNF can encode temporal features of presynaptic neuronal activity. High frequency bursts are more effective in releasing BDNF than continuous depolarization or tonic electrical stimulation [61]. The role of spinal microglia in the development and maintenance of neuropathic pain becomes evermore important. After sciatic CCI spinal microglia is activated by monocyte chemoattractant protein-1 and ATP. The latter induces an influx of extracellular calcium by binding its $P2X_4$ receptor subsequently increasing release and *de novo* synthesis of BDNF in a p38 MAPK-dependent manner. This leads to disinhibition of the DH that is the result of increased excitatory synaptic drive to putative excitatory 'delay' firing neurons in the substantia gelatinosa and decreased synaptic drive to putative inhibitory 'tonic' firing neurons. The depolarizing shift in the anion reversal potential of lamina I neurons that lays at the basis of this phenomenon is the result of a reduction in the expression of the potassium-chloride exporter KCC2. Sensitization of the NMDA receptor additionally contributes to a state of central hypersensitivity. In contrast, there is an upregulation of NPY in damaged large neurons in the CCI model also causing mechanical allodynia. ERK seems to be a main player in this event. A noteworthy observation in the CCI model made by Obata [19] is that the extent of the injury evoked by loose ligatures varies significantly even if performed by one investigator.

Sciatic nerve transection increases BDNF at the site of injury. The source of this BDNF seems to be an increased BDNF production by Schwann cells as well as an intensified anterograde transport of BDNF produced in the sensory neurons of the DRG. In contrast to the sciatic CCI model, in the sciatic nerve transection model BDNF is upregulated in medium to large DRG neurons and in the deeper laminae III and IV of the DH and BDNF expression decreases in small DRG neurons and their projections in the superficial laminae I and II of the DH. Again ERK seems to be the important intracellular transduction pathway in large neurons. There is extensive co-localisation of BDNF and NPY in the large neurons. The diminished BDNF expression in small neurons is caused by diminution in target-derived NGF. Several mechanisms have been postulated for the increase in BDNF expression in large neurons, ranging from depletion in target-derived NGF and/or NT3 to an increase in intracellular calcium concentration

either by direct influx or through the NMDA receptor. Although, it seems unlikely that in the sciatic nerve transection model such an increase in intracellular calcium would occur through calcium channels since Baccei and Kocsis [62] demonstrated a reduction in high-voltage-activated calcium channel currents mainly through a reduction in N-type calcium current. NMDA receptors are present in peptidergic and non-peptidergic primary afferent neurons in the DRG [63]. Nonetheless proof is still needed that extracellular glutamate is present to activate these NMDA-receptors. A causal relationship between increased BDNF expression in sciatic transection models and neuropathic pain is established by experiments in which application of anti-BDNF at the site of nerve transection or i.t., or administration of inhibitors of the intracellular transduction pathway diminish mechanical allodynia.

The spinal nerve ligation model increases BDNF expression in medium to large neurons of the DRG, whereas in uninjured DRG the increase was mainly seen in small to medium neurons and to a lesser extent in large neurons. After injury there was an increase in phosphorylated ERK in large neurons whereas p38 and JNK were activated in small- to medium-sized neurons. In the uninjured level an increase in phosphorylated p38 was noticed in small to medium neurons. An amplified NGF production in the sciatic nerve or L5 spinal nerve seems to be retrogradely transported to the L4 DRG inducing an increase in BDNF production in small- to medium-sized DRG neurons. The mechanism of increased BDNF expression in large neurons in the uninjured DRG needs to be elucidated but possibly large neurons co-expressing TrkA and TrkC receptors may play a key role. A remarkable finding in SNL experiments is that thermal hyperalgesia seems to be mediated by uninjured DRG while mechanical allodynia is mediated by injured DRG.

Spinal nerve transection increases BDNF expression in medium to large DRG neurons as does dorsal or combined dorsal/ventral rhizotomy. Ventral rhizotomy alone results in enhanced BDNF expression in small to medium DRG neurons whereas only lesions distal to the DRG (SNT) lead to an increased BDNF expression in the L4 DRG. Besides neuropathic pain, SNT also gives rise to sympathic basket formation and sprouting around large DRG neurons. Peripherally applied antisera to NGF, NT3 and BDNF attenuate allodynia and sympathic sprouting at different allodynia stages, with BDNF antiserum acting during early and advanced stages but NGF and NT3 antisera acting only during the advanced stage. Blocking BDNF reduces allodynia and sympathic sprouting the longest although the strongest suppression is achieved by antisera to NT3. The findings of the literature review are recapitulated in Table 1.

Table 1. Summary of the literature findings regarding BDNF expression in different animal models of neuropathic pain (CCI: chronic constriction injury, SNL: spinal nerve ligation, SNT: spinal nerve transection, ERK: extracellular signal-regulated protein kinases, p38: p38 mitogen-activated protein kinase, DRG: dorsal root ganglion, DH: dorsal horn, BDNF: brain-derived neurotrophic factor, NGF: nerve growth factor, NT3: neurotrophin 3, Ca^{++}: calcium ion)

Type of nerve lesion	Type of neurons injured	Increased BDNF expression		
		DRG neurons (intracellular transduction pathway)	DH laminae	Mechanism
Sciatic Nerve CCI	medium,large	small, medium (p38)	I, II	Increased NGF production by degenerating Aβ and Aδ fibers, retrogradely transported by intact C fibers
Sciatic Nerve Transection	small, medium, large	medium, large (ERK)	III, IV	Deprivation of NGF in C fibers. Depletion of NGF or NT3 or increased intracellular Ca^{++} in Aδ, Aβ fibers?
L5 SNL	medium, large	medium, large in the L5 DRG (ERK) small, medium in the L4 DRG (p38)	I, II, III, IV	Increased NGF production at the ligation site, transported to the L4 DRG
L5 SNT	small, medium, large	medium, large in the L5 DRG	Undetermined	Deprivation of NGF in C fibers. Depletion of NGF or NT3 or increased intracellular Ca^{++} in Aδ, Aβ fibers?

Although a body of evidence exists for a pronociceptive function of BDNF, contradictory data are found in the literature claiming an antinociceptive effect. In a unilateral CCI of the rat sciatic nerve Cejas [64] succeeded in reversing cold and tactile allodynia and hyperalgesia by lumbar subarachnoid transplantation of BDNF secreting neurons. There was no change in sensory behaviours in a control group. The antinociceptive effects of intrathecal BDNF are thought to arise from BDNF's ability to increase the synthesis and release of serotonin in descending raphe serotonergic/peptidergic neurons or by rescuing central GABA neurons and stimulating GABA release, both providing inhibitory modulation of the DH. Furthermore, using the unilateral sciatic CCI model in female Wistar Furth rats, Eaton [65] succeeded in reversing allodynia and hyperalgesia by adeno-associated viral vector mediated overexpression of BDNF in the spinal cord. They injected the vector into the dorsal spinal gray matter of the first lumbar segment. *In situ* hybridization demonstrated that both dorsal and ventral lumbar spinal neurons contained an intense signal for BDNF mRNA, one week after vector injection. Lever [66] investigated the release of BDNF and GABA in a DH with dorsal roots attached preparation obtained from spinal nerve lesioned male Wistar rats. They demonstrated that BDNF facilitates the release of GABA through activation of TrkB receptors in the dorsal horn. GABA activates the $GABA_A$ receptor found on

large myelinated fibers to mediate GABA-induced depolarization of primary afferent fibers and $GABA_B$ receptor in unmyelinated fibers to mediate GABA-induced inhibition of neurotransmitter release. Contrary to other reports, these investigators found that a single i.t. injection of BDNF relieves thermal hyperalgesia after SNL. This effect was blocked by the GABA receptor antagonist CGP 55445, suggesting this effect was mediated by the release of endogenous GABA from interneurons.

One possible explanation for the conflicting evidence may be interspecies differences in expression of neuropathic pain and BDNF [67]. Even though the levels of expression of BDNF in naive F344, Lewis and Sprague-Dawley rats are very similar, after sciatic CCI, only F344 rats completely recovered from mechanical allodynia, after 28 days. The increase in BDNF mRNA level after CCI was only significant in injured DRG of F344 rats. Moreover, differences in acute inflammatory and neuropathic pain in Lewis and Fischer rats seem to be correlated with the lower levels of circulating corticosterone due to a hypofunctional hypothalamo-hypophyseal adrenal axis [68]. Carr [69] found that the behavioral response of Sprague–Dawley rats to nerve injury is more intense than that of Wistar rats. Also intraspecies disparity between male and female animals in the expression of BDNF and neuropathic pain may cause inconsistency. In addition, differences in the time between the initial nerve perturbation and the examination of DRG cells in different studies presents yet another source of discrepancy because the spontaneous activity induced by nerve injury peaks at 13 days and returns to a lower level after 30 days [70]. Also the loss of thermal hyperalgesia is a normal feature of the loose ligation, although the time at which hyperalgesia disappears, varies [6]. Variations in seemingly similar techniques to induce neuropathic pain may cause different degrees of neuronal damage, as has been demonstrated by ATF3 labeling in a sciatic CCI by Obata [19]. There is also the possibility that the distance between the site of injury and the DRG may affect the intensity of changes seen in cell bodies [71]. In addition, differences in the age of animals used in different studies may have been a factor of variability [55, 72]. Peripheral axotomy promotes much more cell death in the DRG of young animals than in older animals [73]. Finally, Miki [74] demonstrated that the dose of systemically administered BDNF influences the final outcome on neuropathic pain. Whereas low doses of BDNF can suppress mechanical allodynia, high doses have the opposite effect.

2.5 Conclusions

Animal models yielded large amounts of experimental data that indicate the important role of BDNF in neuropathic pain. Yet, these data are not always conclusive because the respective studies differ as to species, gender and age of animals used and because the cell types studied have not been exactly identified. Future studies with more strict experimental paradigms and protocols, may solve this problem. More insight into the

involvement of BDNF in neuropathic pain may emerge from detailed investigation into the differential translation of the multiple BDNF transcripts and their actions on extracellular receptors as well as on intracellular targets [75]. Such studies may provide the basis for the development of BDNF-based drugs for neuropathic pain treatment.

2.6 Abbreviations

ATF3	activating transcription factor 3
BDNF	brain-derived neurotrophic factor
CCI	chronic constriction injury
DH	dorsal horn
DRG	dorsal root ganglion
ERK	extracellular signal-regulated protein kinases
GABA	γ-aminobutyric acid
IR	immunoreactive
i.t.	intrathecally
JNK	Jun N-terminal kinase
KCC2	potassium-chloride co-transporter 2
MAPK	mitogen-activated protein kinase
NGF	nerve growth factor
NPY	neuropeptide Y
NT3	neurotrophin 3
NT4	neurotrophin 4
NT5	neurotrophin 5
P38	p38 mitogen-activated protein kinase
P75NTR	p75 neurotrophin receptor
SNL	spinal nerve ligation
SNT	spinal nerve transection
Trk	tropomyosin-related kinase

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Results supplement published online

BDNF expression in the spinal nerve ligation model

In a spinal nerve ligation model inflammatory mediators and neurotrophins produced by Wallerian degeneration of the ligated L5 and L6 spinal nerve can interact with intact L4 fibers. Ha [1] performed a tight L5-L6 spinal nerve ligation in male Sprague-Dawley rats and found a decrease in BDNF expression in small DRG neurons with an increase in medium and large DRG neurons at the L5 and L6 level. In the ipsilateral L4 DRG small, medium and large DRG neurons showed an increased BDNF-IR. A considerable increase in the expression of BDNF-IR axonal fibers in the medial superficial and deeper laminae of the ipsilateral L4/5 DH was observed. This pattern of BDNF expression resembles more a transection model than a CCI model if we compare it with data obtained from sciatic nerve models. A possible explanation may be found in the tightness with which the ligatures were applied. Normally for CCI injury loose ligations are made hereby causing intraneural edema and constriction of the nerve over days, in this way sparing the smaller C-fibers and injuring only the larger Aβ and Aδ fibers. With tight ligatures it is likely that all fibers are injured, resembling to some extent a transection model. Why small, medium and large neurons in the L4 DRG start to express BDNF is open to discussion. NGF produced by Wallerian degeneration of the L5 and L6 spinal nerve could be retrogradely transported to the L4 DRG, stimulating BDNF production in small and medium neurons, but Shen [2] demonstrated an increased NGF and BDNF mRNA expression in the DRG after a SNL. Identical results concerning BDNF expression in the uninjured L4 DRG in a L5 SNL model were obtained by Fukuoka [3]. The increased BDNF expression mainly took place in TrkA positive small- ($<600\mu m^2$) and medium-sized ($600\text{-}1200\mu m^2$) neurons but also in a subset of large neurons. I.t. administration of a BDNF antiserum attenuated thermal hyperalgesia for a few hours. Furthermore, NGF but not NGFmRNA content increased in L4 DRG. NGFmRNA did increase in the L5 DRG and in the sciatic nerve, indicating that NGF synthesized in the sciatic nerve or the L5 spinal nerve diffuses into the spared L4 spinal nerve. Additionally, local application of a NGF antiserum to the L4 spinal nerve delayed the development of thermal hyperalgesia but did not prevent it. Remarkably, NGF antiserum did not block the increased BDNF expression in the ipsilateral L4 DRG. This is in contrast with the experiment by Obata [4] where NGF antiserum blocked BDNF expression in the L4 DRG after an L5 SNL. Moreover, the molecular processes involved in the manifestation of neuropathic pain in an L5 SNL were investigated. In the injured L5 DRG, the L5 SNL induced the activation of ERK in medium to large neurons and p38 and JNK in small- to medium-sized DRG neurons. In contrast, in the uninjured L4 DRG, an L5 SNL only induced p38 activation in TrkA-expressing small- to medium-diameter C-fiber neurons. I.t. infusions of the MEK1/2 inhibitor U0126 and the JNK inhibitor SP600125 reversed SNL-induced mechanical allodynia, events that could only be blocked by the

p38 inhibitor SB203580. An L5 dorsal rhizotomy made immediately before an L5 SNL prevented the development of mechanical allodynia but was not able to block SNL-induced thermal hyperalgesia indicating that this hyperalgesia originates in the L4 DRG. Further corroborating this evidence is the fact that an L4 dorsal rhizotomy executed after the L5 SNL reversed not only mechanical allodynia but also thermal hyperalgesia. Activation of p38 in the uninjured L4 DRG might therefore be involved in the development of heat hypersensitivity in the L5 SNL model. An L5 SNL induced an increase in BDNF mRNA seen mainly in small- and medium-sized p-38 positive L4 DRG neurons. Treatment with a p38 inhibitor and with NGF antiserum reduced SNL-induced upregulation of BDNF expression in the L4 DRG. These findings suggest that after L5 SNL, NGF synthesized and released in the degenerative nerve fibers acts on nearby sensory fibers along the course of the nerve and induces p38 activation in the adjacent intact small to medium TrkA expressing L4 DRG neurons. This may lead to an increased expression of BDNF and thermal hyperalgesia and mechanical allodynia.

Ten days after an L5 and L6 SNL in male Sprague-Dawley rats, Hayashida [5] found an increase in BDNF content in L5 and L6 (injured) DRG's and in the L4 (uninjured) DRG. No indication as to the size of the neurons concerned was made. There was a coinciding increase in noradrenergic fiber density in the DH at L4, L5 and L6. Although i.t. infusion of anti-BDNF did not change mechanical allodynia, it did reduce noradrenergic axons in the spinal cord as well as the efficacy of 15 µg i.t. applied clonidine on mechanical allodynia, indicating a key role for BDNF in sprouting of noradrenergic fibers in neuropathic pain states.

The summarized data are found in Figure S1.

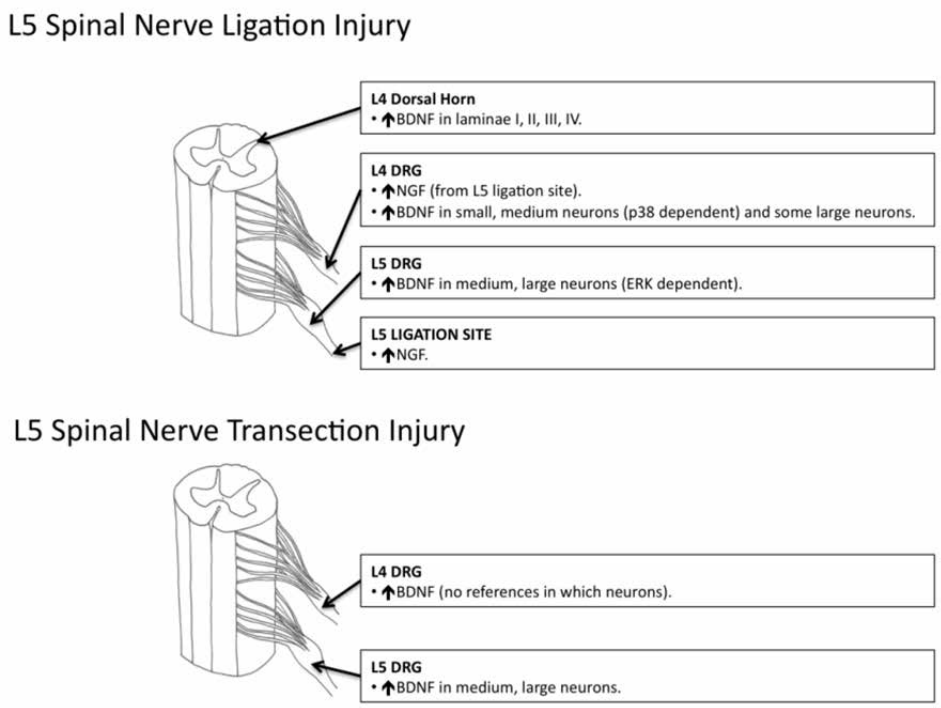

Figure S1. BDNF expression in the spinal nerve ligation and spinal nerve transection model (BDNF: brain-derived neurotrophic factor, DRG: dorsal root ganglion, NGF: nerve growth factor, p38: p38 mitogen-activated protein kinase, ERK: extracellular signal-regulated protein kinases, ↑: increase, ↓: decrease).

BDNF expression in the spinal nerve transection model.

Of all the neuropathic animal models, the spinal nerve transection model is the least researched with regard to BDNF expression. Obata [6] studied the effect of nerve injury site on BDNF expression and neuropathic pain behaviour 7 days after lesion. At the L5 level they performed either a unilateral dorsal rhizotomy, a ventral rhizotomy, a dorsal and ventral rhizotomy or a spinal nerve transection. All procedures induced an increase in BDNF mRNA and protein in the L5 DRG. In the dorsal rhizotomy, dorsal and ventral rhizotomy and spinal nerve transection groups the increase in BDNF-IR was mainly visible in medium and large neurons whereas in the ventral rhizotomy group increased BDNF-IR was mainly seen in small and medium neurons. Mechanical allodynia and heat hypersensitivity were apparent only in the ventral rhizotomy and spinal nerve transection groups, underlining the need for an intact dorsal root for the transportation of BDNF from the medium and large DRG neurons to the DH and the subsequent development of neuropathic pain behaviour. Furthermore, an increase in L4 BDNF-IR was noted only in the L5 spinal nerve transection group, indicating that only a

lesion distal to the L5 DRG can change the phenotype of the intact L4 DRG. Since degenerating fibers initiate a cascade of cellular and molecular events that ultimately lead to an accumulation of chemokines, cytokines and growth factors, the authors speculated that the products of Wallerian degeneration of L5 nerve fibers induced phenotypic changes in the L4 DRG. However, after dorsal and ventral rhizotomy the same products of Wallerian degeneration are produced and should also induce phenotypic changes in the L4 DRG, making this hypothesis incorrect or at least incomplete. In an L5 spinal nerve transection model, Zhou [7] administered antibodies to NGF, BDNF and NT3 in the L5 DRG. Foot withdrawal to Von Frey filaments was reduced compared to controls in all antibody groups. Antibodies to NGF acted during the early phase whereas anti-BDNF and anti-NT3 antibodies were effective during the later phase. No synergistic effect was observed by combining antibodies. The delivery of neurotrophins in normal DRG leads to mechanical allodynia, which occurred fastest when NGF was applied (4 hours) followed by BDNF (3 days). NT3 had only a short-lived effect of 1 day whilst the effect of NGF and BDNF lasted for the whole duration of the experiment (7 days).

In peripheral nerve injury, noradrenergic perivascular axons sprout into the DRG to form baskets around large diameter neurons and are implicated in neuropathic pain after nerve injury [8]. These sprouting fibers are associated with reactive satellite cells that produce NGF, BDNF and NT3. Expression of the p75 nerve growth factor receptor in glia and neurons is required for basket formation but not for the sprouting of sympathetic axons in DRG [9]. Deng [10] administered antisera specific to NGF, NT3, and BDNF intraperitoneally twice a week for 2 weeks in male Sprague–Dawley rats that underwent transection of the L5 spinal nerve. They found reduced sympathetic sprouting and reduced formation of baskets. Foot withdrawal responses to von Frey hair stimuli were attenuated. The effect of anti-BDNF occurred earlier and lasted longer than that of NGF and NT3 antisera. Anti-NT3 is most potent in reducing the sprouting of noradrenergic fibers, followed by NGF and BDNF. Sympathetic sprouting was reduced by about 60%, a similar extent as that blocked by anti-neurotrophins directly delivered to DRG. The summarized data are found in Figure S1.

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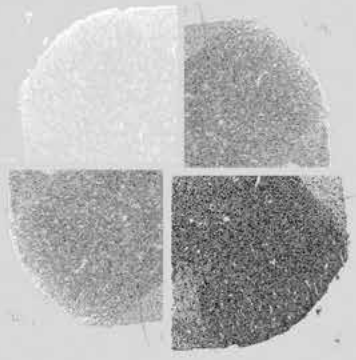

CHAPTER 3

Effects of chronic administration of amitriptyline, gabapentin and minocycline on spinal brain-derived neurotrophic factor expression and neuropathic pain behavior in a rat chronic constriction injury model

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Published in Regional Anesthesia and Pain Medicine, 2013 Mar-Apr;38(2):124-30

Abstract

Background and Objectives
In animal models of neuropathic pain (NP), promising results have been reported with the administration of minocycline, possibly through inhibition of spinal brain-derived neurotrophic factor (BDNF) expression. No data are available on the effect of amitriptyline and gabapentin on spinal BDNF expression. If the mechanism of action of the latter drugs does not involve BDNF inhibition, further clinical research into BDNF is warranted.

Methods
In this placebo-controlled study, we investigated the effects of amitriptyline (5mg/kg), gabapentin (50mg/kg) and minocycline (25mg/kg) twice a day on NP behavior in a sciatic chronic constriction injury (CCI) rat model. Drug treatment started 7 days after CCI and lasted 14 days. At postoperative day 21, spinal BDNF expression in laminae I and II was quantified using immunocytochemistry.

Results
Sciatic CCI resulted in NP behavior throughout the duration of the experiment in the placebo group. When administered for 2 weeks, minocycline ($P \leq 0.001$) and amitriptyline ($P \leq 0.05$), but not gabapentin, reduced thermal hyperalgesia. None of these drugs reduced mechanical allodynia. As opposed to amitriptyline and gabapentin, 2 weeks treatment with minocycline reduced BDNF-immunoreactivity ($P \leq 0.05$) in the ipsilateral dorsal horn.

Conclusions
Minocycline and amitriptyline both reduce NP behavior in a sciatic CCI injury rat model, but only minocycline reduces spinal BDNF indicating different modes of action of these 2 drugs. The observed actions of minocycline closely fit the clinical needs for the treatment of NP.

3.1 Introduction

Neuropathic pain (NP) arises as a direct consequence of a lesion or disease affecting the somatosensory system. First-line pharmacological treatment is based either on increasing central nervous system serotonin and norepinephrine levels by tricyclic antidepressants and selective serotonin and norepinephrine reuptake inhibitors or on decreasing glutamate and substance P release by blocking the α_2-δ subunit of voltage-gated calcium channels with anticonvulsant drugs. However, despite these therapeutic modalities, NP often persists [1]. Moreover, adverse effects of these drugs often limit or prohibit their use. Consequently, there is an urgent need for novel targets and drugs to improve current NP treatment.

Neurotrophins can be such targets. Their superfamily consists of nerve growth factor, brain-derived neurotrophic factor (BDNF) and neurotrophins 3 and 4/5. Each family member exerts its function through its own high affinity tropomyosin-related receptor kinase (Trk) and a shared low affinity p75 neurotrophin receptor. During the past decade, BDNF was studied extensively in various animal models for NP and emerged as a modulator of central and peripheral nociceptive responses. Enhanced BDNF expression by neurons and microglia in the spinal sensory matrix favors nociception through suppression of inhibitory mechanisms within the dorsal horn and enhancement of excitatory synaptic output [2]. Moreover, there is a close temporal relationship between the appearance and disappearance of thermal hyperalgesia and spinal BDNF concentration [3].

Amitriptyline and gabapentin are both first-line drugs used in the treatment of NP [4]. In order for BDNF to be a useful new target for the treatment of NP we must first ascertain that these existing drugs have no effect on BDNF expression. Should amitriptyline and gabapentin already significantly decrease BDNF expression, then the clinical usefulness of BDNF as a new target in the treatment of NP would be questionable, and research into new compounds to inhibit BDNF expression would seem pointless. On the other hand, if amitriptyline and gabapentin do not affect BDNF expression, a combination of drugs with different modes of action could prove helpful in the treatment of NP. However, to date their effect on spinal BDNF expression has not been studied. Minocycline, a second-generation tetracycline, improves several neurodegenerative conditions and decreases NP behavior in various animal models [5, 6], possibly via a BDNF-dependent mechanism, yet minocycline has not found its way into clinical practice for pain relief.

The first aim of this study was to examine the effect of amitriptyline, gabapentin and minocycline on spinal BDNF expression and NP behavior using the well-established chronic sciatic nerve constriction injury (CCI) rat model for NP and quantitative immunocytochemistry in the dorsal horn. The second aim was to mimic the clinical situation by commencing drug therapy 7 days after CCI, instead of preemptively, and by administering these drugs for 2 weeks. Most animal studies investigating the effects

of amitriptyline, gabapentin and minocycline on NP involve preemptive study designs, with the start of drug therapy before induction of NP and the duration of therapy limited to 7 days maximum [7-9].However, this is not a fair reflection of the clinical situation as NP is a chronic condition and most patients present themselves well after NP has emerged.

3.2 Methods

3.2.1 Ethical considerations

The study was conducted in a manner that does not inflict unnecessary pain or discomfort upon the animal, as outlined by the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (1996), prepared by the National Academy of Sciences' Institute for Laboratory Animal Research. All handlings were approved by the animal ethics committee of the Radboud University Nijmegen.

3.2.2 Animal care

Forty-six male Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany), weighing 340±3 g, were randomly allocated to five groups: 'minocycline' (n=9), 'amitriptyline' (n=8), 'gabapentin' (n=9), 'control' (n=8) and 'sham' (n=6). Animals were pair-housed in plastic cages (Makrolon type III; Tecniplast, Buguggiate, Italy) for 7 days to acclimatize and, upon CCI- or sham-surgery, during the next 3 weeks of experimentation, in a temperature- and humidity-controlled environment, on a 12/12 h light-dark cycle (lights on: 6:30 AM, light intensity: 200 lux), with *ad libitum* access to tap water and soy-free rodent chow.

3.2.3 CCI-surgery

Sciatic CCI was performed as described by Bennett and Xie [10]. In short, on experiment day 0, all rats were anesthetized with isoflurane (induction 5%, maintenance 2.5%)/nitrous oxide (65-67.5%) and the left sciatic nerve was exposed at the mid-thigh level proximal to the trifurcation by blunt dissection of the biceps femoris muscle. Next, 4 chromic catgut 5-0 ligatures (Catgut, Markneukirchen, Germany) were loosely applied, 1 mm apart, around the left sciatic nerve. Finally, the overlying muscle was sutured with Vicryl 5-0 (Johnson & Johnson, St. Stevens-Woluwe, Belgium) and the skin closed with autoclips (7.5 x 1.75 mm; Rudolf Medizintechnik, Fridingen, Germany).

Sham-operated animals underwent the same surgery but without applying nerve ligation.

3.2.4 Drug treatment

All drugs were dissolved in 0.9% NaCl. CCI rats received either 25 mg/kg body weight minocycline orally, 5 mg/kg amitriptyline subcutaneously (SC), 50 mg/kg gabapentin SC or 5 ml 0.9% NaCl/kg SC (controls) twice a day, at 7 AM and 7 PM, from day 7 till day 21. Shams received 5ml 0.9% (wt/vol) NaCl/kg SC. Drug doses were chosen according to the lowest concentrations previously reported to have significant effects on NP behavior [11-13].

3.2.5 Behavioral tests

On the day before surgery (BL; pre-surgery baseline) and on days 7, 14 and 21, 3 behavioral tests were carried out. First, rats were tested for cold hyperalgesia on a cold plate apparatus (Ugo Basile, Comerio, Italy) consisting of an acrylic cylinder (diameter 19 cm, height 31 cm) fixed to a stainless steel plate with a constant temperature of 4°C. The numbers of left and right hind paw lifts were counted during a 5 minute period. Secondly, mechanical hyperalgesia was assessed with the Von Frey test [14], placing a rat on the mesh-wired floor of a plastic box (20 x 16 x 14 cm), for 30 minutes. Force-calibrated Von Frey monofilaments (Somedic AS, Stockholm, Sweden) were applied perpendicularly to the plantar surface of the hind paw. Brisk withdrawal or paw flinching were considered as positive responses. In the absence of a response, a filament of next greater force was applied. Each filament was tested 5 times. The filament that elicited 4 positive responses defined the threshold for mechanical allodynia. Third, heat hyperalgesia was tested with the plantar test as described by Hargreaves et al. [15]. After habituating rats in the mesh-wire box for 15 minutes, a heat stimulus was applied to the left or right hind paw, and the time till paw lifting was measured. Cutoff time was 20 seconds to prevent tissue damage. All tests were performed during the light period.

3.2.6 Immunocytochemistry

All animals were killed on day 22 for quantitative immunocytochemistry. They were perfused transcardially under deep isoflurane anesthesia, with 100 ml 0.1 M sodium phosphate-buffered saline (PBS; pH 7.4) for 10 minutes, followed by 200 ml 4% ice-cold paraformaldehyde in PBS for 20 minutes. Next, L5 spinal cord sections (thickness: 10µm) were postfixed in paraformaldehyde at 4 °C for 24 hours. In brief, sections were pre-treated with 1% H_2O_2 in PBS for 15 minutes to minimize endogenous peroxidase activity, and then treated with 0.5% Triton X-100 in PBS, for 30 minutes, blocked in 2%

normal donkey antiserum, for 1 hour, and incubated in primary rabbit anti-BDNF serum (1:250; Cat Nr: ANT-010, Alomone, Jerusalem, Israel), for 16 hours. This was followed by 4x10-minute washes in PBS, and 2-hour incubation in secondary anti-rabbit serum in a dilution of 1:200 (Cy3-conjugated anti rabbit IgG; Cat No: 711-165-152; Jackson Immunoresearch Laboratories; West Grove, PA, USA). The high specificity of the anti-BDNF serum was confirmed by preabsorbing the antiserum with synthetic BDNF, which totally abolished immunostaining. In addition, omission of the primary antiserum completely prevented immunoreaction in all cases.

3.2.7 Morphometry

Sections were examined with a TCS-SP2-AOBS confocal laser-scanning microscope with a 20x/0.7 UV dry lens (Leica Microsystems, Wetzlar, Germany). Counts of BDNF-immunoreactive structures in laminae I and II of the left (ipsilateral) and right dorsal horn were made on digital images taken at 1024x1024 dpi in the 3 most medial transversal sections of the spinal cord, 75 µm apart, and processed with ImageJ 1.44 software (National Institutes of Health, Bethesda, MA, USA). Earlier studies had shown that BDNF is present in both dorsal horns of untreated rats and does not increase in the contralateral dorsal horn upon CCI-surgery of the left sciatic nerve [16, 17]. The difference in BDNF-immunoreactive counts in the ipsilateral (left) and contralateral (right) dorsal horn served as a measure for the effect of CCI on spinal BDNF expression in the left dorsal horn.

3.2.8 Statistics

An experimenter unaware of the surgical and pharmacologic treatments of the animals performed all data acquisition. Statistical analyses were carried out using Prism 5.0d (GraphPad Software, San Diego, CA, USA). For each parameter, data were averaged for animals within an experimental group and expressed as means $\pm$ standard error of the mean (SEM). Analysis of variance was used to examine behavioral changes in an animal group over time, as well as differences between drug-treated and control groups at the various sampling times. This was followed by Tukey's *post hoc* test. Immunocytochemistry data were analyzed with Student's unpaired 1-tailed t-test followed by Kolmogorov-Smirnov's *post hoc* analysis. All analyses were preceded by tests for normality (Shapiro and Wilk, 1965) and homogeneity of variance (Bartlett's Chi-square test; Snedecor and Cochran, 1989). The threshold of significance was at $\alpha = 5\%$.

3.3 Results

3.3.1 Induction of neuropathic pain by CCI

The data from the behavioral studies are presented in Figures 1 and 2. Before surgery (BL; presurgery baseline) none of the rats in any group showed any sign of NP, neither in any of the behavioral tests for cold and heat hyperalgesia or for mechanical allodynia.

In shams, no signs of NP behavior were observed throughout the experiment (Figs. 1A-C). In shams, the frequency of left hind paw lifts (number in 5 minutes) on the cold plate BL (0.5±0.3) remained low and not significantly different from that on day 7 (0.5±0.3), day 14 (2.8±2.8) and day 21 (0.7±0.4). A similar situation held for the latency (seconds) until hind paw withdrawal in the plantar test (day 7: 17.4±1 seconds; day 14: 16.8±0.9 seconds; and day 21: 17.6±1 seconds *vs.* BL 17.4±1 seconds) and for the force needed for hind paw withdrawal with Von Frey filaments (day 7: 16.3±0.6 g; day 14: 16.0±0.8 g; and day 21: 15.5±0.8 g *vs.* BL: 16.3±0.6 g).

In contrast, control animals that underwent CCI surgery followed by administration of 0.9% NaCl, unmistakably exhibited NP behavior in all 3 tests (Fig. 1A-C). Lift frequency on the cold plate was dramatically increased on day 7 (19.6±3.1; $P \leq 0.001$), day 14 (22.1±2.3; $P \leq 0.001$) and day 21 (16.6±3.8; $P \leq 0.01$) when compared with BL (0.3±0.3). Latency until left hind paw withdrawal decreased significantly for the duration of the experiment (day 7: 10.5±1.5 seconds; $P \leq 0.001$; day 14: 10.6±0.9 seconds; $P \leq 0.001$; and day 21: 8.8±0.9 seconds; $P \leq 0.001$ *vs.* BL: 17.2±0.9 seconds) as did the force needed for left hind paw withdrawal with Von Frey filaments (day 7: 14.1±0.3 g; $P \leq 0.001$; day 14: 13.3±0.3 g; $P \leq 0.001$; and day 21: 13.3±0.6 g; $P \leq 0.001$ *vs.* BL: 16.8±0.4 g).

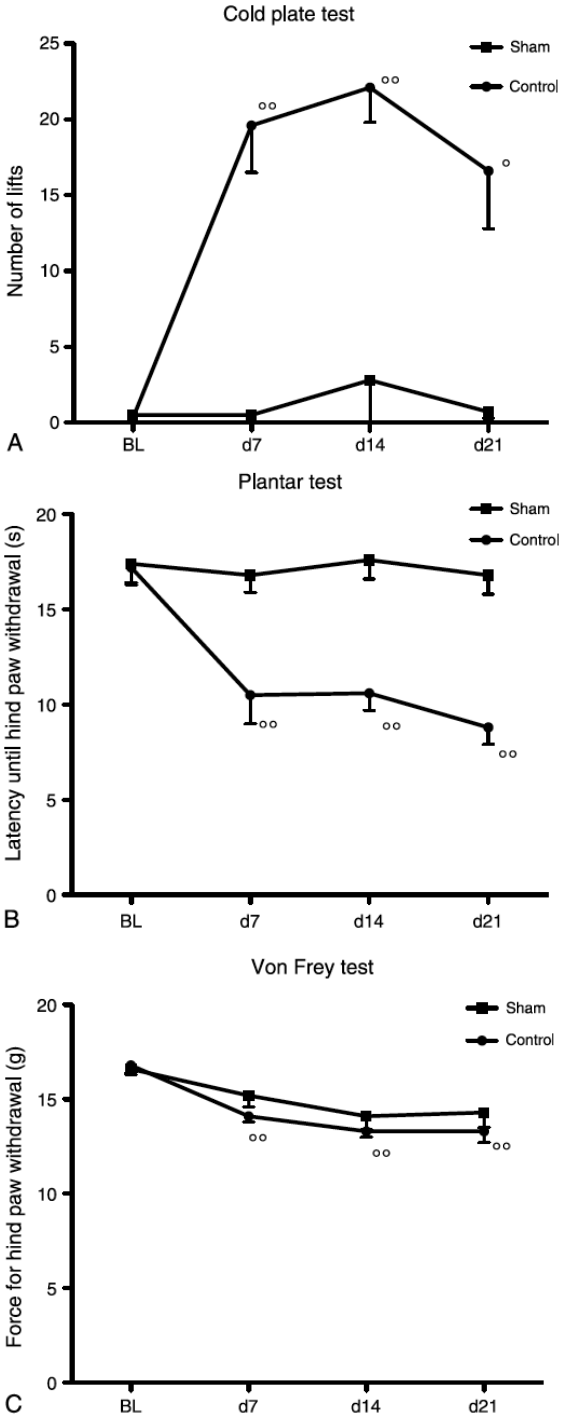

Figure 1. (A) Number of left hind paw lifts in 5 minutes, in the cold plate test; **(B)** latency till withdrawal of the left hind paw in the plantar test; and **(C)** force needed for withdrawal of this paw in the Von Frey test, at BL (pre-surgery baseline), day 7 (start drug administration) and at day 14 and day 21 (after 7 and 14 days of drug administration, respectively). Means ± SEM. Sham (no sciatic CCI); n=6, control (sciatic CCI, 0.9% NaCl injection); n=8. ° $P \leq 0.01$ and °° $P \leq 0.001$ compared with day 7 in the same group.

In the drug-treated groups, CCI also resulted in clear NP behavior in all 3 tests (Figs. 2A-C). In the minocycline, amitriptyline and gabapentin groups there was a clear increase in lift frequency on day 7 compared with BL (17.4±0.6 *vs.* 0.4±0.3; $P \leq 0.001$; 21.5±2.6 *vs.* 0.5±0.3; $P \leq 0.001$ and 17.2±1.7 *vs.* 1.0±0.9; $P \leq 0.001$, respectively). In the plantar test, time till left hind paw lift on day 7 decreased considerably compared with BL in the minocycline (14.1±1.4 *vs.* 18.2±0.6; $P \leq 0.05$), amitriptyline (11.0±1.3 *vs.* 17.4±0.8; $P \leq 0.01$) and gabapentin (10.2±1.7 *vs.* 16.9±0.4; $P \leq 0.05$) group. Finally, the force needed for left hind paw lift with Von Frey filaments decreased in all 3 drug treated groups on day 7 compared with BL (minocycline: 15.2±0.6 *vs.* 16.6±0.3; $P \leq 0.05$; amitriptyline 13.9±0.6 *vs.* 16.0±0.6; $P \leq 0.05$; gabapentin 14.1±0.3 *vs.* 16.9±0.4; $P \leq 0.001$). At no time point was any effect of CCI found on the unoperated right hind paw.

3.3.2 Effect of drug treatment

There was no statistical difference in the parameters of the behavioral tests at day 7 between controls, amitriptyline-, gabapentin- or minocycline-treated rats. Control animals clearly exhibited cold and heat hyperalgesia and mechanical allodynia, which persisted throughout the experiment (Figs 1A-C). Whereas on day 21 there was a spontaneous reduction in the number of lifts on the cold plate, this did not reach statistical significance compared with day 7. In the plantar test and Von Frey test there were no signs of spontaneous recovery.

Behavioral results from the minocycline, amitriptyline and gabapentin groups are presented in Figs. 2A-C. Minocycline-treated animals demonstrated progressively decreasing cold hyperalgesia in the cold plate test during the 2 weeks of drug therapy. The number of lifts on the cold plate was significantly reduced on day 14 (9.2±1.9; $P \leq 0.05$) and day 21 (6.6±1.3; $P \leq 0.001$) when compared with day 7 (17.4±2.6). Also on days 14 and 21 when compared with control animals, rats in the minocycline group lifted considerably less (22.1±2.3 *vs.* 9.2±1.9; $P \leq 0.01$ and 16.6±3.8 *vs.* 6.6±1.3; $P \leq 0.05$ respectively). The latency until left hind paw withdrawal in the plantar test was reduced on day 21 when compared with control (8.8±0.9 seconds *vs.* 14.3±1.4 seconds; $P \leq 0.05$). In the Von Frey test minocycline failed to reverse mechanical allodynia at any time point (day 14: 14.1±0.7 g; $P > 0.05$; day 21: 14.3±0.8 g; $P > 0.05$ vs. day 7: 15.2±0.6 g).

Amitriptyline progressively diminished cold hyperalgesia during the experiment. On day 21 the number of lifts on the cold plate was lower than on day 7 (14.4±2.1 *vs.* 21.5±2.6; $P \leq 0.05$). However, in the plantar and Von Frey tests amitriptyline-treated animals showed no improvement in NP behavior.

Although there was a clear trend for gabapentin to reduce the number of lifts on the cold plate, the different means did not reach statistical significance. The same held for heat hyperalgesia and mechanical allodynia during the 2 weeks of drug treatment. At no time point was any effect of drug treatment found on the unoperated right hind paw.

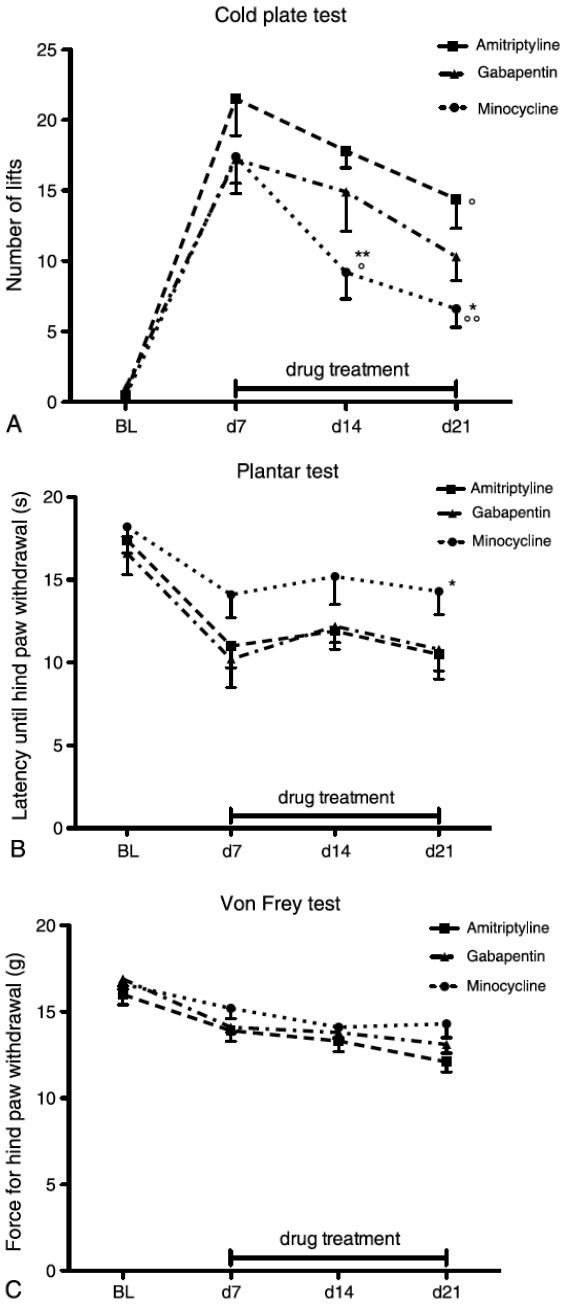

Figure 2. (A) Number of left hind paw lifts in 5 min, in the cold plate test; **(B)** latency till withdrawal of the left hind paw in the plantar test; and **(C)** force needed for withdrawal of this paw in the Von Frey test, at BL (pre-surgery baseline), day 7 (start drug administration) and at day 14 and day 21 (after 7 and 14 days of drug administration, respectively). Minocycline-treated (n=9), amitriptyline-treated (n=8), gabapentin-treated (n=9) rats. Means ± SEM. * $P \leq 0.05$ and ** $P \leq 0.01$, compared with the corresponding day in the control group (n=8). ° $P \leq 0.05$ and °°$P \leq 0.001$ compared with day 7 in the same group.

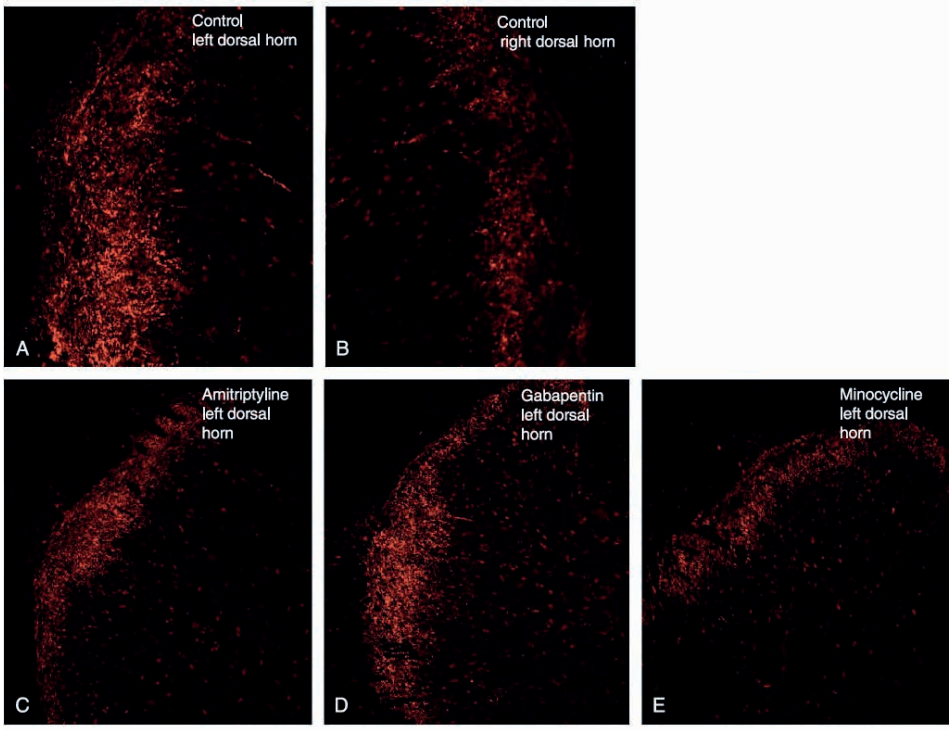

Figure 3. Immunocytochemistry of BDNF-immunoreactive structures in the dorsal horn of a control (no drug treatment) CCI rat, with stronger immunoreactivity in the left horn (A) than in the right one (B). After drug treatment immunoreactivity is lower in minocycline group (E) than in amitriptyline (C) and gabapentin (D) groups.

3.3.3 BDNF-immunoreactivity in the dorsal horn

Sections revealed clear BDNF immunoreactivity in the dorsal horns of control- and drug-treated animals, which was mainly located in laminae I and II (Fig. 3). When compared with control (918±287), only minocycline reduced the number of BDNF-immunoreactive structures in the left dorsal horn (369±104; $P \leq 0.05$). Although the number of BDNF-immunoreactive structures was lower in both the amitriptyline (529±125) and gabapentin (641±155) group compared to the control group, this did not reach statistical significance (Fig. 4).

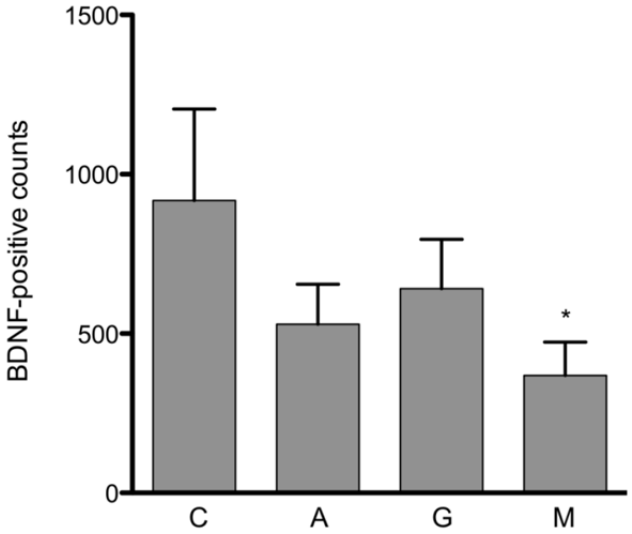

Figure 4. Quantification of BDNF-immunoreactivity in the left dorsal horn. Means ± SEM. C indicates control (n=4); A, amitriptyline (n=4); G, gabapentin (n=6); M, minocycline (n=5). * $P \leq 0.05$, compared with control group.

3.4 Discussion

In the present study, we demonstrated the effectiveness of minocycline and amitriptyline in reducing thermal hyperalgesia when started 7 days after a sciatic CCI. The effects were more pronounced in the minocycline group than in the amitriptyline group whereas neither of these drugs could reverse CCI-induced mechanical allodynia. Surprisingly, when administered 7 days after CCI, gabapentin had no effect on either thermal hyperalgesia or mechanical allodynia. After 2 weeks of drug treatment, minocycline but not amitriptyline or gabapentin reduced BDNF-immunoreactivity in the ipsilateral dorsal horn.

Our behavioral data on the effects of minocycline on NP concur with earlier short-term (7 days) studies where preemptive administration of minocycline was effective in reducing NP behavior in rodent sciatic CCI [7-9]. However, the present study is the first to demonstrate a long-lasting effect (up to 21 days post-injury) of minocycline when administered 7 days after CCI instead of preemptively. This profile neatly fits the clinical situation because patients consult their physicians after the emergence of NP, making preemptive drug administration impossible, and because chronic administration is often required. However, to date, no clinical trials have been conducted to assess the effect of minocycline on NP in humans. Although effectively reducing thermal hyperalgesia in our CCI rats, minocycline had no effect on mechanical allo-

dynia when administered 7 days post-injury. A similar outcome was observed in a spinal nerve ligation model [18] where administration of minocycline from day 7 forwards had no appreciable effect on mechanical allodynia. However, when administered on day 1 or day 3, minocycline substantially reversed mechanical allodynia (47.7% and 24.9% reduction of paw withdrawal threshold, respectively) indicating that the therapeutic window for minocycline to reverse mechanical allodynia after CCI surgery is limited to 3 days, whereas for thermal hyperalgesia this window is at least 7 days. The mechanism underlying the differential actions of minocycline on mechanical *vs.* thermal hyperalgesia deserves further research.

In previous animal studies preemptively administered amitriptyline was effective in reducing NP in a spared nerve injury model and in a spinal nerve ligation model [11, 19]. Yet, clinical data about the preemptive effect of amitriptyline are conflicting because trials revealed positive as well as negative outcomes [20, 21]. In our study, amitriptyline was effective in reducing cold hyperalgesia when started 7 days after CCI. However, because amitriptyline was less effective than minocycline, minocycline may be more appropriate for use in a clinical setting where drug application starts after emergence of NP. Nevertheless, like minocycline, amitriptyline was unable to reverse mechanical allodynia in our study. Pradhan et al. [22] found identical results on mechanical allodynia in a CCI and spinal nerve ligation model when amitriptyline was administered 7 days post-injury. It is possible that the therapeutic window for amitriptyline for reversal of mechanical allodynia is, as for minocycline, smaller than 7 days.

Here we have demonstrated that gabapentin only reveals a trend towards reducing cold hyperalgesia without reaching statistical significance. In other animal studies gabapentin was able to reduce NP behavior when given preemptively and post-injury [12, 22]. The results of our animal study are in line with a multicenter clinical trial investigating the effects of gabapentin in traumatic nerve injury pain. After gabapentin treatment for 5 weeks, the authors found no decrease in mean pain intensity score. This could point to a greater clinical relevance of our study paradigm compared to short-term studies with preemptive drug administration to investigate drug effects on traumatic nerve injury [23].

The reduced NP behavior after 14 days of minocycline administration was accompanied by markedly reduced spinal BDNF expression in the ipsilateral dorsal horn. These findings are in line with those of Miletic and Miletic [3] who found a positive time correlation between spinal BDNF concentration and emergence and disappearance of thermal hyperalgesia in rats. Further studies confirming the importance of BDNF in the pathophysiology of NP are those in which BDNF was sequestered by intrathecal application of BDNF-antiserum or of TrkB/fc fragments or by inhibiting the TrkB receptor by K-252a thus preventing the development of thermal hyperalgesia in animal CCI models [24, 25]. These data suggest that minocycline exerts its analgesic effects through a BDNF-dependent mechanism whereas for amitriptyline other mechanisms prevail [11, 19]. In our experiment, we did not explore the mechanism by which

minocycline reduces BDNF expression in the dorsal horn, but the literature suggests an involvement of mitogen-activated protein kinase pathways [18].

In conclusion, this study adds to our current knowledge of BDNF expression, NP, and its treatment, by demonstrating that amitriptyline and gabapentin, drugs currently used for the treatment of NP, do not exert their analgesic effect through a BDNF-dependent pathway as opposed to minocycline. Moreover, our study reveals that minocycline more than amitriptyline has a long-lasting effect on NP behavior not only when administered pre-emptively but also when administered after the establishment of NP. This finding is clinically relevant since most patients consult their physician after the development of NP. Future investigations should focus on the combined effects of drugs with different modes of action and on validating the results of the animal studies involving minocycline in clinical practice.

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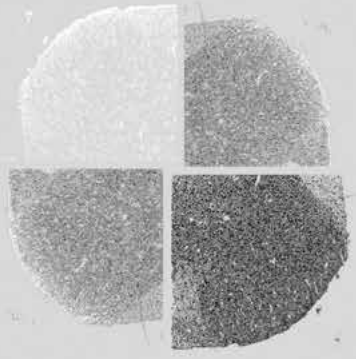

CHAPTER 4

The effect of minocycline on lumbar radicular neuropathic pain: a randomized, placebo-controlled, double-blind clinical trial with amitryptiline as a comparator

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Submitted

Abstract

Background
Less than 50% of patients experience sufficient pain relief with current drug therapy for neuropathic pain. Minocycline shows promising results in rodent models of neuropathic pain but was not yet studied in humans with regard to the treatment of neuropathic pain.

Methods
In this randomized, double-blind, placebo-controlled clinical trial, patients with subacute lumbar radicular pain received placebo, amitriptyline 25mg or minocycline 100mg once a day (n=20 per group) for 14 days. Primary outcome measure was the pain intensity in the leg as measured by a numeric rating scale (NRS) ranging from 0 to 10 on days 7 and 14. Secondary outcome measures were reduction of neuropathic pain symptoms in the leg as determined with the DN4 questionnaire, consumption of rescue medication and adverse events on days 7 and 14.

Results
Sixty patients were randomized and included in an intention-to-treat analysis. After 14 days, patients in the minocycline and amitriptyline groups reported a reduction of 1.47 (95% confidence interval: 0.16-2.83, P=0.035) and 1.41 (95% confidence interval 0.05-2.78, P=0.043), respectively in NRS score compared to the placebo group. No differences were seen in DN4 values at any time point during treatment between the 3 groups. The rate of adverse events in the amitriptyline group was 10% vs. none in the minocycline and placebo groups. No differences were noted in the consumption of rescue medication.

Conclusion
These short-term outcomes suggest that minocycline and amitriptyline are equally effective in treating lumbar radicular neuropathic pain and warrant additional larger trials.

4.1 Introduction

Chronic pain imposes a heavy burden on patients and society. With an annual cost of US$ 560 billion, the expenditure of pain in the United States exceeds these of cancer, heart disease and diabetes combined [1]. Neuropathic pain, defined as pain arising from a lesion within the somatosensory nervous system [2], is one of the most debilitating forms of chronic pain. Although neuropathic back and leg pain are the most common forms of neuropathic pain [3], they are the least researched with regard to drug efficacy. Amitriptyline for instance, is a tricyclic antidepressant and first-line drug in the treatment algorithm of neuropathic pain [4] but was never studied in patients with lumbar radicular pain. Furthermore, efficacy of a drug for a particular type of neuropathic pain cannot be extrapolated to another type of neuropathic pain [5-7] and, moreover, first-line therapies for neuropathic pain are often accompanied by considerable side effects, limiting their usability [8-10]. Consequently, there is a strong need for studies with accepted first-line drug therapies in patients with lumbar radicular pain as well as for novel drugs to treat neuropathic pain.

Minocycline is a semisynthetic tetracycline with antibiotic action against a wide range of gram-positive, gram-negative and atypical micro-organisms. It attenuates mechanical allodynia and thermal hyperalgesia in rodent models of neuropathic pain when administered pre-emptively [11, 12] and post-injury [13], mainly through microglial inhibition and decreased expression of brain-derived neurotrophic factor [11, 13]. Beneficial effects of minocycline have been observed in the neurological and functional recovery of patients with stroke [14], spinal cord injury [15] and multiple sclerosis [16]. A first indication that minocycline can improve neuropathic pain, was recently obtained by Syngle et al [17] in patients suffering from polyneuropathy associated with type 2 diabetes. With the present study we aim to confirm and extend their observation by testing the effect of minocycline on lumbar radicular neuropathic pain. Since in humans, minocycline is readily absorbed by the gastro-intestinal tract [18] and easily crosses the blood-brain barrier with cerebrospinal fluid levels ranging between 25 and 30% of serum concentrations [19], oral administration of this drug is suitable to treat central nervous system diseases.

In the present randomized, double-blind, placebo-controlled clinical trial with amitriptyline as a comparator, we are the first to test the hypothesis that minocycline reduces neuropathic pain in patients suffering from lumbar radicular pain. Since we performed this trial as a proof of concept study, we choose the smallest number of participants needed based on our power analysis together with a relatively short follow-up period of 2 weeks in order not to withhold patients from other treatments. The primary objective of this study was to analyze the effect of minocycline on pain intensity as compared to placebo and amitriptyline. Secondary outcome measures included assessment of neuropathic pain symptoms, consumption of rescue medication, and possible adverse events. This study was part of a larger clinical trial that

included determination of plasma and serum concentrations of brain-derived neurotrophic factor in patients with lumbar radicular pain (clinicaltrials.gov number NCT01869907).

4.2 Methods

4.2.1 Study design and settings

In this single centre, randomized, double-blind, placebo-controlled, clinical trial with amitriptyline as an active comparator, we investigated the effect of minocycline on neuropathic lumbar radicular pain. The trial was conducted at the Ziekenhuis Oost-Limburg in Genk, Belgium and was approved by the local ethics committee (Commissie voor Medische Ethiek) and registered at clinicaltrials.gov (trial number: NCT01869907). The study started in September 2011 and ended in August 2013 when the objective of 60 enrolled patients had been reached.

4.2.2 Participants

Patients were recruited by means of physician referrals to our tertiary multidisciplinary pain clinic at the Ziekenhuis Oost-Limburg in Genk, Belgium. Eligible patients presented with lumbosacral radicular pain radiating into the leg below the knee caused by disc herniation, spinal canal stenosis or failed back surgery syndrome. Patients were included only if the level of the pathology on CT- or MR-imaging correlated with the dermatome in which they indicated their leg pain and if the leg pain was predominant over the back pain (NRS score for leg pain > NRS score for back pain). The dermatomal distribution of the leg pain was determined with the adapted dermatomal map from Wolff et al [20]. The neuropathic nature of the leg pain was determined by a validated Dutch translation of the douleur neuropathique (DN4) questionnaire (cut-off ≥ 4) [21]. The in- and exclusion criteria are presented in Table 1. All patients gave written informed consent before inclusion in the study.

Table 1. In- and exclusion criteria of the study

Inclusion criteria:	Exclusion criteria
Age 18 to 80 years	Diabetic, alcoholic or drug induced polyneuropathies
Neuropathic lumbar radicular pain caused by:	Depression or psychiatric comorbidity affecting pain sensation
Lumbar disc herniation, spinal canal stenosis or failed back surgery syndrome (epidural fibrosis) confirmed by CT- or MR-imaging	Use of antidepressants
Level of pathology on imaging studies correlates with dermatomal distribution in the leg	Fibromyalgia and chronic fatigue syndrome Pregnancy Spinal cord damage Malignancy Allergy for minocycline or amitriptyline

CT: computer tomography; MR: magnetic resonance.

4.2.3 Interventions

Sixty patients were randomized in a 1:1:1 ratio to receive once daily, during 2 weeks, either placebo (starch; Fagron, Waregem, Belgium), amitriptyline 25mg (Aca Pharma, Waregem, Belgium) or minocycline 100mg (ABC Chemicals, Wouters-Brakel, Belgium). Patients were instructed to take the study medication in the morning with a glass of water one hour before or one hour after breakfast, to prevent interference of food with gastro-intestinal absorption with the study drug [18]. Continuation of paracetamol or non-steroidal anti-inflammatory drugs was permitted during the trial period on the condition that patients were on a stable dose for at least 1 week prior to enrolment. Otherwise, the only pain medications permitted during the trial period were the study drug and rescue medication. Rescue medication consisted of 50mg tramadol (Tradonal Odis; Meda Pharma, Brussels, Belgium) with a maximum of 3 intakes daily and 6h between consecutive ingestions. During the trial period patients completed 3 study visits: a baseline visit and visits after 7 and 14 days during which the pain intensity in the leg measured by a 11-point numeric rating scale (NRS) and DN4 score was obtained, and a supply for 1 week of trial (7 capsules) and rescue medication (21 tablets) was provided. During visits 2 and 3 patients were asked if they experienced any adverse events. Drug logs were kept to record the amount of rescue medication the patients had consumed during the past week and to record if the patients had taken the entire amount of investigational drugs.

4.2.4 Outcome measures

The primary outcome measure was the effect on pain intensity in the leg. Patients were asked to rate their average leg pain during the past 24h on an 11-point NRS with 0 indicating no pain and 10 the worst pain imaginable [22]. Secondary outcome measures were changes in neuropathic pain symptoms in the leg (burning pain sensation, cold painful sensation, electric shocks, paresthesia, 'pins and needles' sensation, numbness, itching pain sensation, hypoesthesia to touch, hypoesthesia to pinprick and mechanical allodynia, with a score of 1 awarded for every symptom present) as measured with the DN4 [21], changes in consumption of rescue medication and adverse events. The assessments were made at baseline (visit 1) and after 7 (visit 2) and 14 days (visit 3) for the NRS and DN4 scores and during visits 2 and 3 for consumption of rescue medication and adverse events. In order to detect adverse events, patients were asked on days 7 and 14 of the trial if they had experienced side-effects during the past week attributable to the study drugs.

4.2.5 Blinding and randomization

A hospital pharmacist prepared study kits containing the study drug and rescue medication for each patient for the entire duration of the study and randomly assigned a number to each kit ranging from 1 to 60. Each kit contained 2 white vials labeled 'study medication', each vial containing 7 capsules and 2 white vials labeled 'rescue medication', each vial containing 21 tablets of 50mg tramadol. The encryption key linking the numbers of each kit to its content was safeguarded by the pharmacist until the end of the study and was unknown to all the outcome assessors. Placebo, amitriptyline and minocycline were encapsulated in identical opaque white capsules so that patients and study staff were unable to visually distinguish their contents. Upon inclusion, patients were randomized by assigning them a study kit number by a computerized random number generator.

4.2.6 Statistical methods

A decrease of 2 or more points on an 11-point NRS has been shown to be moderately clinically meaningful [23]. In order to detect this difference with a standard deviation of 1.7 [24] and a power of 90% with a significance level set $\alpha = 0.05$, we calculated that a sample size of 16 patients per group would be needed [25]. In order to compensate for an estimated dropout rate of 20% we included 20 patients per group. An intention-to-treat analysis was performed with none of the patients excluded because of missing data. To determine the effect of placebo, amitriptyline 25mg and minocycline 100mg on NRS and DN4 scores, we used a linear regression model with time in weeks, the baseline value of the outcome, dummies for the active interventions and an interac-

tion between time and the intervention dummies as covariates. The 2 interaction terms were entered to estimate the change over time for the different interventions. A random intercept model was used to take into account the statistical dependence of the measurements. A mixed model analysis of variance with Bonferroni's *post-hoc* test was used to assess changes in consumption of rescue medication. Finally, a cumulative proportion responder analysis [26], which displays the level of response at all possible cut-off points, was performed for NRS and DN4 scores on day 14. The cut-off points are the percent change in NRS pain intensity or DN4 score respectively, on day 14 compared to day 1 (e.g. 50% signifies that the NRS score or DN4 score has halved on day 14). Data are presented as means ± standard error of the mean (SEM). Statistical analyses were carried out using IBM SPSS version 20, release 20.0.0.1 (Armonk, NY, USA).

4.3 Results

4.3.1 Study population

Sixty patients were randomized, 20 to each treatment group. Nine patients (3 patients per study arm) discontinued the trial in the first week, 2 because of violation of the study protocol (one patient took the study medication for 2 days and then switched to non-steroidal anti-inflammatory drugs, the other patient stopped study medication after 2 days without giving a reason), 2 because of adverse effects (both in the amitriptyline group) and 5 patients withdrew their informed consent. In total 51 patients (17 per treatment arm) completed the study (Fig. 1). The baseline characteristics were similar across the 3 study arms (Table 2).

4.3.2 Primary outcome

All patients were included in the regression analysis. After 2 weeks of therapy, patients in the minocycline and amitriptyline groups reported a reduction of 1.47 (95% confidence interval: 0.16-2.83, P=0.035) and 1.41 (95% confidence interval 0.05-2.78, P=0.043) in NRS score, respectively, compared to the placebo group. There was no statistically significant difference in NRS score between the minocycline and amitriptyline group after 2 weeks of therapy (Fig. 2 panel A). The cumulative proportion responder analysis at day 14 showed more responders in the minocycline and amitriptyline group than in the placebo group for all cut-off points (Fig. 2 panel B). The number needed to treat (NNT) for moderate (≥30%) and substantial (≥50%) clinically important improvements in NRS score [27] are 3 and 6 for minocycline and 3 and 4 for amitriptyline, respectively.

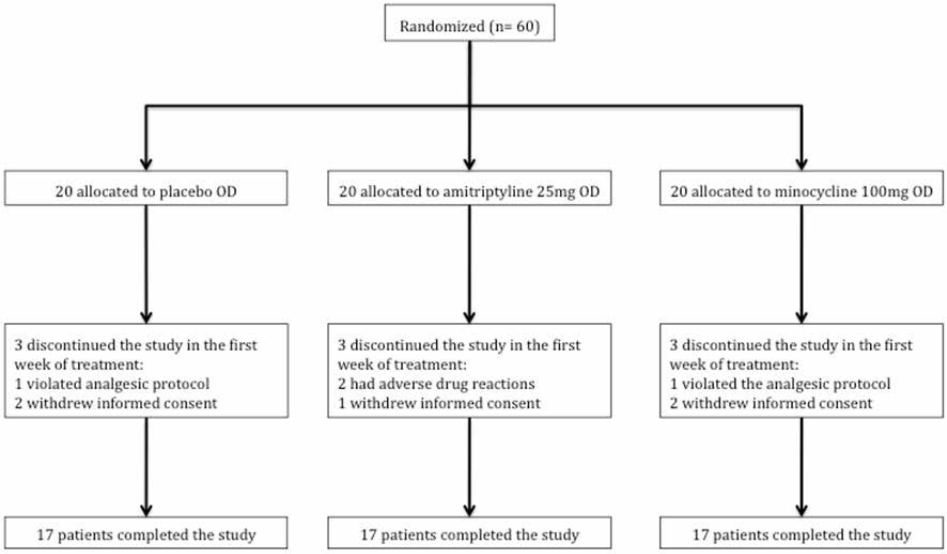

Figure 1. CONSORT flowchart.
OD: once daily.

Table 2. Demographic data and baseline characteristics of the patients

Characteristic	Placebo (n=20)	Amitriptyline (n=20)	Minocycline (n=20)
Age - yr	51±3	50±3	47±3
Sex - no. (%)			
Male	9 (45)	13 (65)	11 (55)
Female	11 (55)	7 (35)	9 (45)
Culprit nerve root - no. (%)			
L3	0 (0)	2 (10)	1 (5)
L4	3 (15)	1 (5)	7 (35)
L5	8 (40)	6 (30)	8 (40)
S1	9 (45)	11 (55)	4 (20)
Cause of neuropathic pain - no. (%)			
Disc herniation	16 (80)	16 (80)	17 (85)
Spinal canal stenosis	4 (20)	2 (10)	1 (5)
Failed back surgery syndrome	0 (0)	2 (10)	2 (10)
Duration of pain - mo	2.8±0.4	3.2±0.6	2.5±0.3
NRS score	7.4±0.3	6.9±0.4	7.1±0.4
DN4 score	4.2±0.4	4.3±0.4	4.9±0.3

Plus-minus values are means±SEM. NRS: numeric rating scale ranging from 0 to 10 for leg pain; higher scores indicate more pain. DN4: douleur neuropathique questionnaire, scores ranging from 0 to 10; higher scores indicate more neuropathic pain symptoms are present. Cut-off for the presence of neuropathic pain is a score ≥ 4.

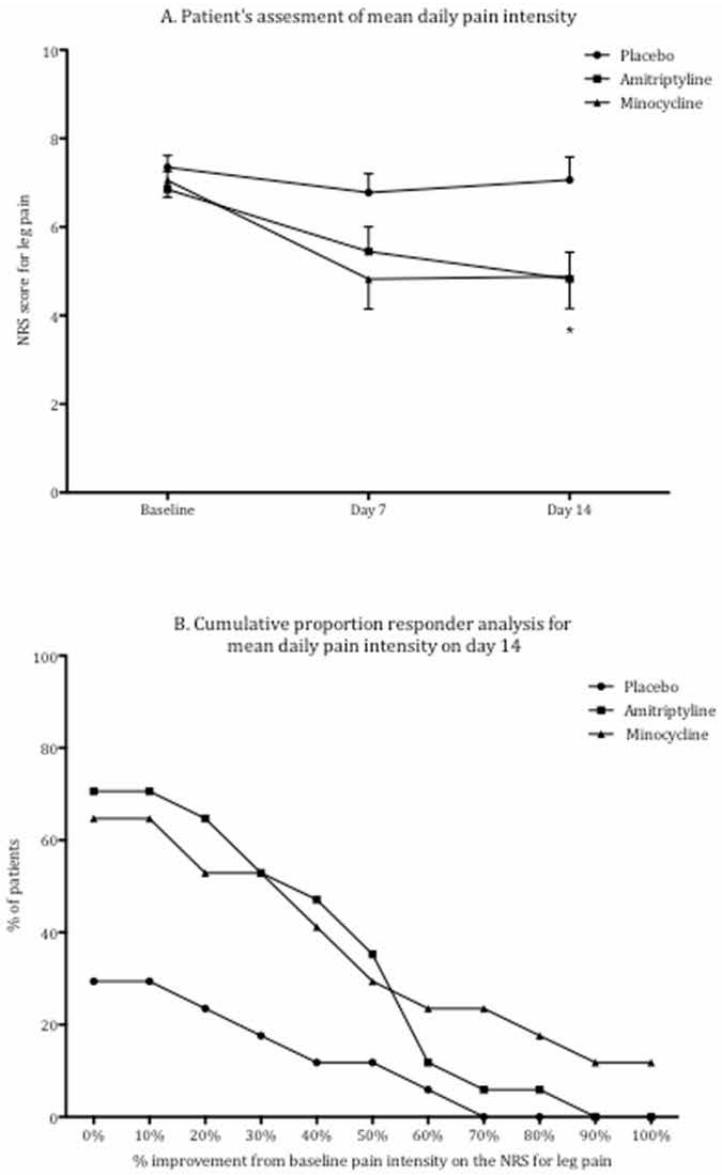

Figure 2. Primary outcome measures
Panel A. Patient's assessment of pain intensity
NRS: numeric rating scale ranging from 0 to 10 for leg pain. Higher scores indicate more pain.
* P<0.05 for NRS score in the minocycline and amitriptyline group compared to placebo on day 14. Panel B. Cumulative proportion responder analysis for mean daily pain intensity in the leg on day 14 as measured by the NRS. X-axis: level of response (% improvement) in mean daily pain intensity from baseline NRS score on day 14. Y-axis: proportion of patients (%) that equal or exceed the level of response.

4.3.3 Secondary outcomes

With respect to DN4 scores, 2 weeks therapy resulted in decrease of 1.26 points (95% confidence interval: -0.01-2.25, P=0.053) and 0.35 points (95% confidence interval: -0.8-1.48, P=0.54) in the amitriptyline and minocycline group, respectively, compared to placebo. Cumulative proportion responder analysis of neuropathic symptoms also showed closely matched responder curves for amitriptyline, minocycline and placebo, indicating no obvious differences between the 3 treatment groups (Fig. 3). There were no statistically significant differences in the consumption of rescue medication between the 3 groups at any time point (Table 3).

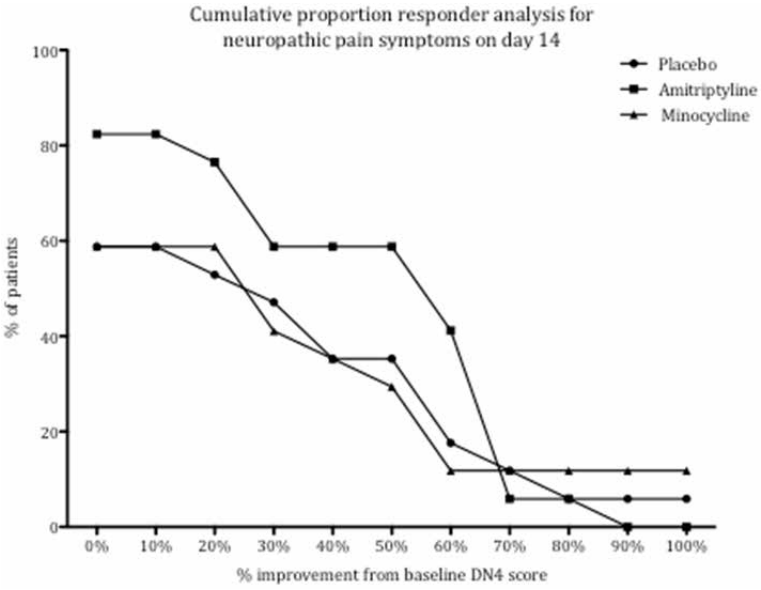

Figure 3. Cumulative proportion responder analysis for neuropathic pain symptoms in the leg on day 14 as measured by the DN4. X-axis: level of response (% improvement) in neuropathic pain symptoms from baseline on day 14. Y-axis: proportion of patients (%) that equal or exceed the level of response.

Table 3. Secondary outcome measures

	DN4 score			Rescue medication	
	Baseline	Day 7	Day14	Week 1	Week 2
Placebo	4.2±0.4	3.7±0.5	3.6±0.6	12±2	12±2
Minocycline	4.9±0.3	4.2±0.5	3.9±0.5	11±2	14±2
Amitriptyline	4.3±0.4	2.9±0.4	2.2±0.3	9±2	9±2

Plus-minus values are means±SEM. DN4: douleur neuropathique questionnaire;
higher scores indicate more neuropathic pain symptoms are present. Cut-off for the presence of neuropathic pain is a score ≥ 4, scores ranging from 0 to 10.
Rescue medication is the number of 50mg tramadol tablets taken over the course of the past week.
No statistically significant differences in DN4 score or amount of rescue medication were noted between the three treatment groups at any time point.

4.3.4 Adverse events

In the amitriptyline group, 2 out of 20 patients (10%) reported adverse events within the first week of treatment. One patient complained of nausea, vomiting and a general unwell feeling, and a second patient developed a rash. All symptoms disappeared after discontinuation of the study medication. No adverse events were reported in the placebo and minocycline groups.

4.4 Discussion

The results from this study suggest that minocycline and amitriptyline improve lumbosacral radicular pain for at least 2 weeks and, furthermore, that treatment with minocycline is associated with less adverse events compared to amitriptyline. After 2 weeks of treatment, minocycline and amitriptyline reduced pain intensity by 1.47 and 1.41 points on the NRS, respectively. This result was not caused by differences in the amount of rescue medication taken in the different groups. The placebo response for moderate improvement in NRS score was 18% in our study, approximating the placebo response of other neuropathic pain trials in a Cochrane review [9]. With this result we not only support the conclusion made by Syngle et al [17] that minocycline reduces neuropathy but extend it to lumbar radicular pain.

To date, no other studies are available on the use of minocycline for the primary treatment of lumbar radicular neuropathic pain in humans. In one study, researchers investigated the preventive effect of minocycline on persistent pain after lumbar discectomy [28]. Minocycline was administered perioperatively for 8 days, but failed to reduce the incidence of low back and leg pain 3 months after surgery. However, a subgroup analysis in the latter study suggested that patients with similar symptoms as

the patients in our study (predominant deep spontaneous neuropathic leg pain) might benefit from minocycline treatment. Another study concerned the effect of a single dose of minocycline on neuropathic pain induced by an intradermal capsaicin injection in patients with unilateral sciatica, which showed no significant effect on NRS score [29]. The negative results in both these studies could be related to the timing and duration of minocycline administration since in our study the antinocicepive effect of minocycline only became apparent after 2 weeks of treatment.

In our study, minocycline shows a reduction in pain intensity with similar NNT's compared to amitriptyline, a first-line drug for the treatment of neuropathic pain. We found no randomized studies concerning amitriptyline and radicular neuropathic pain, but our NNT values for amitriptyline match those found in a systematic review by Moore *et al.* where the effects of amitriptyline on painful diabetic neuropathy, post-herpetic neuralgia, post-stroke pain and fibromyalgia were pooled [9]. Our data are in contrast with a study by Khomori *et al.* [5] where nortriptyline, an active metabolite of amitriptyline and also a first-line drug for the treatment of neuropathic pain, failed to improve chronic lumbar radicular pain. Two important differences between our study and the study of Khomori can provide an explanation for these opposing outcomes of two almost identical drugs. First, there is a different time interval to treatment: in our study patients had a mean duration of pain of approximately 3 months while in the study by Khomori and colleagues the median duration of pain was 5 years. It is known that pain becomes less responsive to treatment the longer it persists [30]. Whether this effect is due to consolidation of pathophysiologic processes, rendering neuropathic pain less responsive to drug treatment over time or due to acquired overlying psychosocial issues and psychopathology in patients with chronic pain, resulting in lower response rates for treatments remains to be elucidated. Second, there is a different timing in administration of the tested drugs: in our study amitriptyline was administered in the morning whereas nortriptyline in the study by Khomori was administered in the evening. Chronopharmacology studies showed significant higher serum concentrations of amitriptyline when administered in the morning than in the evening due to a higher absorption rate constant and a shorter time to peak concentration [31]. This can also account for the fact that we found a significant clinical effect with relative low doses of amitriptyline.

In a randomized controlled trial [32] and in a systematic review [33], combination therapy for neuropathic pain (tricyclic antidepressants or antiepileptics combined with opioids and nortriptyline or gabapentin alone or combined, respectively) was found beneficial over monotherapy. However, the usability of combination therapy is often limited due to overlapping side-effects of the drugs. The absence of adverse events associated with minocycline treatment (*vs.* 10% in the amitriptyline group) in our study, suggests that minocycline could be useful in combination therapy. The adverse event rate of 10% we noticed in the amitriptyline group, was lower than the 64%

previously reported in a systematic review [9] probably because of the short duration of our trial and the relatively low dose of amitriptyline we used.

The limitations of our study are related to the short follow-up period and a relative small number of participants. However, the optimal timing of, and duration for the treatment of neuropathic pain in humans with minocycline is unknown and our power calculation was effective in predicting the amount of patients needed to detect a significant drop in pain intensity. The dosages of minocycline (100mg daily) and amitriptyline (25mg daily) we used, were lower than reported in other studies [9, 28] but in view of the fact that neuropathic pain often requires long-term treatment, the lowest dose of a drug with a clinically ample effect should be used, especially because 25mg amitriptyline already impairs daily functions such as car driving [34]. Since potentially serious side-effects have been reported with the use of minocycline [35], larger studies with more detailed safety assessments are needed to determine the definite risk-benefit profile of this drug for the treatment of neuropathic pain. Finally, future studies may benefit from keeping a pain diary of NRS scores over a 24h period.

In conclusion, our short-term results suggest that minocycline and amitriptyline are equally effective in treating lumbar radicular neuropathic pain and, moreover, that treatment with minocycline is associated with fewer side-effects. This study warrants additional clinical trials with larger patient populations, longer follow–up and more intricate designs (e.g. crossover) to evaluate long-term outcome and safety and to study the effects of minocycline on other neuropathic pain conditions.

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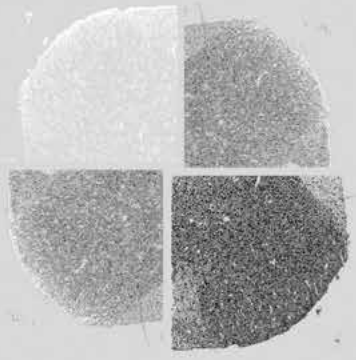

CHAPTER 5

Occipital neuralgia: evidence-based interventional pain medicine according to clinical diagnoses

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Published in Pain Practice, 2010 Mar-Apr;10(2):137-44

Abstract

Occipital neuralgia is defined as a paroxysmal shooting or stabbing pain in the dermatomes of the nervus occipitalis major and/or nervus occipitalis minor. The pain originates in the suboccipital region and radiates over the vertex. A suggestive history and clinical examination with short-term pain relief after infiltration with local anesthetic confirms the diagnosis. No data are available about prevalence or incidence of this condition. Most often trauma or irritation of the nervi occipitales causes the neuralgia. Imaging studies are necessary to exclude underlying pathological conditions. Initial therapy consists of a single infiltration of the culprit nervi occipitales with local anesthetic and corticosteroids (2 C+). The reported effects of Botulinum toxin A injections are contradictory (2 C±). Should injection of local anesthetic and corticosteroids fail to provide lasting relief, pulsed radiofrequency treatment of the nervi occipitales can be considered (2 C+). There is no evidence to support pulsed radiofrequency treatment of the ganglion spinale (dorsal root ganglion) C2. As such this should only be done in a clinical trial setting. Subcutaneous occipital nerve stimulation can be considered if prior therapy with corticosteroid infiltration or pulsed radiofrequency treatment failed or provided only short-term relief (2 C+).

5.1 Introduction

This review on occipital neuralgia is part of the series "Evidence-based Interventional Pain Medicine According to Clinical Diagnoses". Recommendations formulated in this article are based on "Grading strength of recommendations and quality of evidence in clinical guidelines" described by Guyatt et al., and adapted by van Kleef et al. in the editorial accompanying the first article of this series [1,2](Table 1).

The latest literature update was performed in November 2009.

Table 1. Summary of evidence scores and implications for recommendations

Score	Description	Implications
1 A +	Effectiveness demonstrated in various RCT's of good quality. The benefits clearly outweigh risk and burdens.	Positive recommendation
1 B +	One or more RCT's with methodological weaknesses, demonstrate effectiveness. The benefits clearly outweigh risk and burdens.	
2 B +	One or more RCT's with methodological weaknesses, demonstrate effectiveness. The benefits closely balanced with risk and burdens.	
2 B ±	Multiple RCT's with methodological weaknesses, yield contradictory results better or worse than the control treatment. Benefits closely balanced with risk and burdens, or uncertainty in the estimates of benefits, risk and burdens.	Considered, preferably study-related
2 C +	Effectiveness only demonstrated in observational studies. Given that there is no conclusive evidence of the effect, benefits closely balanced with risk and burdens.	
0	There is no literature or there are case reports available, but these are insufficient to suggest effectiveness and/or safety. These treatments should only be applied in relation to studies.	Only study-related
2 C −	Observational studies indicate no or too short-lived effectiveness. Given that there is no positive clinical effect, risk and burdens outweigh the benefit.	Negative recommendation
2 B −	One or more RCT's with methodological weaknesses, or large observational studies that do not indicate any superiority to the control treatment. Given that there is no positive clinical effect, risk and burdens outweigh the benefit.	
2 A −	RCT of a good quality which does not exhibit any clinical effect. Given that there is no positive clinical effect, risk and burdens outweigh the benefit.	

RCT: randomized controlled trial

The International Headache Society (IHS) defines occipital neuralgia as paroxysmal shooting or stabbing pain in the dermatomes of the nervus occipitalis major or nervus occipitalis minor [3]. The pain originates in the suboccipital region and radiates over the vertex. Hypo- or dysesthesia in the affected area can accompany the pain. Pressure over the nervus occipitalis major or minor usually elicits the pain.

No data are available about the prevalence or incidence of occipital neuralgia. The nervus occipitalis major is more frequently involved (90%) as compared to the nervus occipitalis minor (10%). In 8.7%, both nervi occipitales are responsible for the neuralgia [4].

Often damage or irritation of the nervus occipitalis major or minor is the cause of the neuralgia. Vital et al. [5] described 2 bends that divide the course of the nervus occipitalis major into 3 parts. The nervus occipitalis major is formed by the ramus dorsalis of the C2 nerve root. The first part runs between the origin of the nerve and the musculus obliquus capitis inferior underneath which the nerve makes its first bend in a medial direction. The second part of the nerve runs cranially between the musculus semispinalis capitis on the one side and the musculus obliquus capitis inferior, musculus rectus capitis posterior and the musculus rectus capitis anterior on the other side. When perforating the musculus semispinalis capitis toward the surface, the nerve makes its second bend in a lateral direction. The third part of the nerve runs further laterally where the aponeurosis of the musculus trapezius is perforated and the nerve begins its subcutaneous course. The nervus occipitalis major usually branches after perforating the aponeurosis.

There are various potential causes of irritation: vascular, neurogenic, muscular and osteogenic.

Vascular:

- Irritation of the nerve roots C1/C2 by an aberrant branch of the arteria inferior posterior cerebelli (posterior inferior cerebellar artery) [6]
- Dural arteriovenous fistula at the cervical level [7]
- Bleeding from a bulbocervical cavernoma [8]
- Cervical intramedullar cavernous hemangioma [9]
- Giant cell arteritis [10-12]
- Fenestrated arteria vertebralis pressing on C1/C2 nerve roots [13]
- Aberrant course of the arteria vertebralis [14]

Neurogenic:

- Schwannoma in the area of the craniocervical junction: schwannoma of the nervus occipitalis [15,16]
- C2 myelitis [17]
- Multiple sclerosis [18]

Muscular/tendinous [19]

Osteogenic:

- C1/C2 arthrosis, atlantodental sclerosis [20]
- Hypermobile arcus posterior of the atlas [21]
- Cervical osteochondroma [22]
- Osteolytic lesion of the cranium [23]
- Exuberant callus formation after fracture C1/C2 [24]

5.2 Diagnosis

5.2.1 History

Patients complain of a shooting or stabbing pain in the neck radiating over the cranium. Constant pain can persist between the paroxysms. The pain can be perceived in the retro-orbital area caused by overlap of the C2 dorsal root and the nucleus trigeminus pars caudalis [25]. Vision impairment/ocular pain (67%), tinnitus (33%), dizziness (50%), nausea (50%), congested nose (17%) can be present because of connections with cranial nerves VIII, IX, and X and the cervical sympathicus [26].

5.2.2 Physical examination

Upon clinical examination, hypo- or dysesthesia in the area of the nervus occipitalis major or minor as well as tenderness to pressure over the course of the nervus occipitalis major or minor can be observed. A positive Tinel's sign (pain upon percussion over the nerve) can be present.

Clinical presentation and a temporary improvement with a local anesthetic diagnostic block of the nervus occipitalis major and/or minor confirms the diagnosis [3]. False-positive results occur with migraine and cluster headaches [27].

5.2.3 Additional tests

Radiography is recommended to rule out underlying pathologies. Open-mouth X-ray of the cervical spine shows possible arthritis of the C2 facet joints. A computer tomography scan of the craniocervical junction is indicated in case of suspected neoplastic or degenerative osseous pathology. It is important to note that degenerative changes of the cervical spinal column do not necessarily correspond with the symptoms the patient is presenting. Magnetic resonance imaging is ideal for visualizing disorders of the cervical and occipital soft tissues.

5.2.4 Differential diagnosis

Tumors, infection and congenital anomalies (Arnold-Chiari malformation) should be ruled out. Occipital neuralgia can be mistaken for migraine, cluster headache, tension headache and hemicrania continua. Other structures may cause similar pain such as the cervical facet joints (C2-C3), osteoarthritis of the atlanto-occipital of atlanto-axial joint**,** giant-cell arteritis and tumors of the cervical spinal column [28].

5.3 Treatment options

5.3.1 Conservative management

Conservative treatment focuses on reducing secondary muscle tension and on improving posture. Pharmacological treatment may include tricyclic antidepressants and antiepileptics (carbamazepine, gabapentin and pregabalin).

5.3.2 Interventional management

Infiltration of the nervi occipitales with local anesthetic and corticosteroids
The most common site to infiltrate the nervus occipitalis major is along its course, where the nerve penetrates the aponeurosis of the musculus trapezius. Here the nerve is most often constricted [29]. Vital et al. found that this site is located on a line that connects the middle of the 2 ears, 3.18 cm from the midline [5]. Becser et al. found that the subcutaneous path of the nerve starts at the level of the intermastoid line, 1.3 cm from the midline [30]. Loukas et al. investigated the best site to infiltrate the nervus occipitalis major based on external landmarks [31]. On average, the nervus occipitalis major is situated 3.8 cm lateral from the midline and one quarter of the distance along a line connecting the protuberantia occipitalis externa to the mastoid (or 2 cm lateral and 2 cm inferior to the protuberantia occipitalis externa). This is in accordance with the findings of Vital [5]. Natsis et al. defined the optimal site for infiltration of the nervus occipitalis major 1.5 cm lateral and 2 to 2.5 cm inferior to the protuberantia occipitalis externa [32]. Great variability in the course of the nervus occipitalis major is described [33].

In a small (n=10) retrospective study by Kuhn et al., the nervus occipitalis major was infiltrated with corticosteroids after a positive test block with bupivacaine [26]. The authors found pain relief less than 1 week in 10% of patients; 1 week in 30%; 2 weeks in 30%; 1 month in 10% and more than 2.5 months in 20% of patients. Hammond et al. found a short-term effect (less than 1 week) in 64% of the patients after 1 infiltration with local anesthetics, 36% of the patients had an effect lasting longer than 1 month [4].

Botulinum toxin A infiltrations

Injections with Botulinum toxin type A in 6 patients relieved the sharp, shooting pain associated with occipital neuralgia, yet had no effect on the dull, aching pain [34]. Quality-of-life measures exhibited some improvement. No significant reduction in pain medication was demonstrated. However, in a retrospective case series of 6 patients with occipital neuralgia Kapural et al. were able to demonstrate a pain reduction after injection of Botulinum toxin A (visual analog scale, VAS, declined from 8±1.8 to 2±2.7) as well as an improvement in the pain disability index [35]. The mean duration of pain relief averaged 16.3±3.2 weeks.

Pulsed radiofrequency treatment of the nervi occipitales

To date 1 case report and 1 prospective trial have been published concerning pulsed radiofrequency (PRF) treatment in occipital neuralgia [36,37]. Both used 20-millisecond bursts with a frequency of 2 Hz and a maximum temperature of 42°C for 4 minutes to the nervi occipitales. In the case report the patient showed 70% pain relief lasting 4 months. After recurrence of pain, the treatment was repeated with again 70% pain relief lasting 5 months. Of the 19 patients included in the prospective trial 68.4%, 57.9% and 52.6% reported an improvement of 50% or more 1, 2 and 6 months after PRF treatment, respectively. The mean VAS score before treatment was 7.5 (standard error of the mean, SEM, ±0.4) and declined to 3.5 (SEM±0.8), 3.5 (SEM±0.7), and 3.9 (SEM±0.8) at 1, 2 and 6 months, respectively (P<0.001, P<0.001, P=0.002, respectively). There was a statistically significant improvement in the use of medication and in quality-of-life parameters.

PRF treatment of the C2 ganglion spinale (Dorsal Root Ganglion, DRG)

A prospective audit looked into the effects of PRF treatment adjacent to the cervical ganglion spinale (DRG). In 4 of the 18 patients, the procedure was carried out at C2 level as a treatment for headache. Two of these 4 patients had a long-term effect (18 and > 24 months), whereas no improvement was observed in the other 2 patients [38].

Subcutaneous neurostimulation of the nervi occipitales

Weiner and Reed first reported 13 patients who underwent 17 implant procedures for medically refractory occipital neuralgia. With follow-up ranging from 18 months to 6 years, good to excellent results were seen in 12 of 13 patients defined by pain relief greater than 50% and requiring little or no pain medications. The thirteenth patient was explanted following resolution of the symptoms [39].

Slavin et al. [40] carried out a trial stimulation in 14 patients with therapy resistant occipital neuralgia. A definitive neurostimulator was implanted subcutaneously in 10 patients who had a reduction in pain of greater than 50%. After a mean follow-up of 22 months, 70% of the patients still had good results. This confirms the results of an earlier study by Slavin et al. [41] where 13 of 18 patients experienced >50% pain

reduction as a result of test stimulation with a favorable follow-up effect, lasting 28 months, in 85% of the patients. Other studies also report comparable results [42,43].

5.3.3 Complications of interventional management

Although infection and bleeding are possible complications of any percutaneous technique, these have only been reported with subcutaneous lead implantation for neurostimulation together with lead migration, hardware erosions, electrode fractures, disconnections, and sepsis [44]. One case report describes sudden unconsciousness due to an inadvertent subarachnoidal injection through an os occipitale defect after craniotomy [45]. Other possible side-effects of occipital nerve infiltrations include temporary dizziness and gait uncertainty, injection site soreness, bradycardia, and focal alopecia [46-49].

5.3.4 Evidence for interventional management

Technique	Evaluation
Single infiltration of the nervi occipitales with local anesthetic and corticosteroids	2 C +
Pulsed radiofrequency treatment of the nervi occipitales	2 C +
Pulsed radiofrequency treatment of the cervical ganglion spinale (DRG)	0
Subcutaneous stimulation of the nervi occipitales	2 C +
Botulinum toxin A injection	2 C ±

5.4 Recommendations

A single infiltration of the nervus occipitalis major with local anesthetic and corticoids can be considered for the treatment of occipital neuralgia. The effects of Botulinum toxin A infiltrations are contradictory. PRF treatment of the nervus occipitalis can be considered if infiltration with local anesthetic and corticoids fail to provide sufficient pain relief. PRF treatment of the ganglion spinale (DRG) C2 or C3 is only recommended in a clinical trial setting. Subcutaneous nerve stimulation can be considered in severe disabling pain unresponsive to other treatments. Considering its relatively high cost and the invasive nature of the treatment, occipital nerve stimulation should be considered later in the treatment algorithm and should be performed in experienced centers.

5.4.1 Clinical practice algorithm

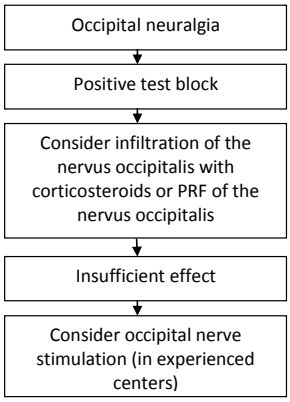

Figure 1. Treatment algorithm of occipital neuralgia

5.4.2 Techniques

Infiltration of the nervus occipitalis major and minor
The infiltration sites of the nervus occipitalis major and minor are determined by external landmarks as described by Vital and Becser (see Figure 2) [5,30]. The needle (22 gauge) is introduced until there is bone contact or until paresthesia is elicited. Subsequently, the needle is slightly withdrawn, and the local anesthetic and corticosteroids are injected.

PRF treatment of the nervus occipitalis major and minor
The puncture site (22 gauge needle, 5 cm, 1 cm active tip) is located according to the external landmarks described by Vital and Becser [5,30]. The thermocouple is introduced after perforation of the skin. The nervus occipitalis major and minor are located with a 50 Hz, 0.5 V current until the patient reports paresthesia in the dermatomes of the nervus occipitalis major or minor. Subsequently, a PRF treatment (45 V, 20 milliseconds, 2 Hz) lasting 120 seconds with a maximum temperature of 42°C is performed twice.

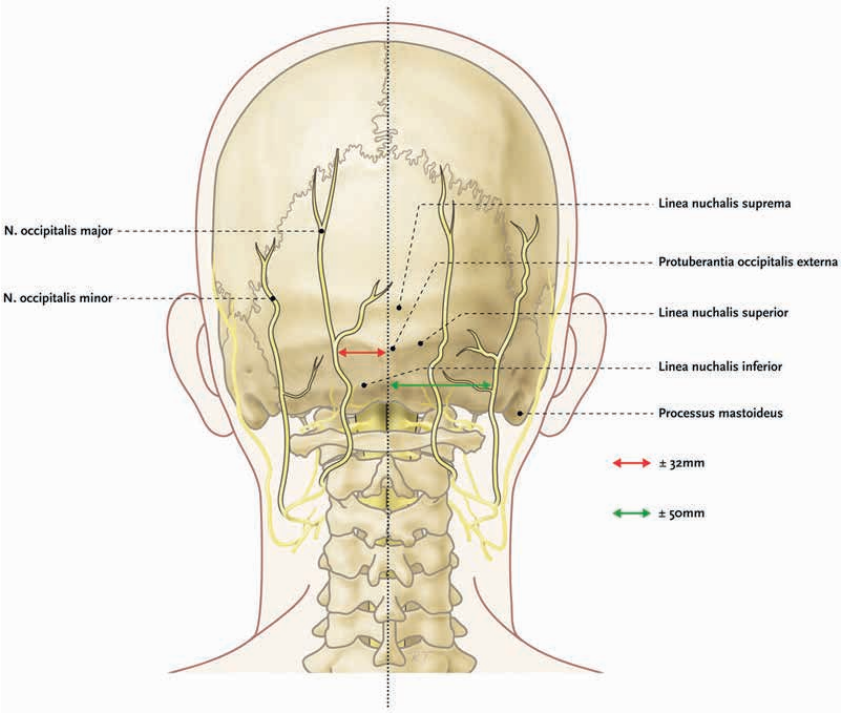

Figure 2. Landmarks for injection of the nervi occipitales. *Illustration: Rogier Trompert, Medical Art.*

Occipital neurostimulation

The first stage of the procedure (the trial) is performed under mild sedation and local anesthesia to monitor the patient's feedback and ensure the most optimal coverage of the painful areas by the stimulation. The patient is placed in a prone position. After disinfection and sterile draping, a curved needle is inserted at the upper lateral end of the neck and advanced toward the midline of the craniovertebral junction along the contour of the C1 arcus vertebralis under fluoroscopic guidance, crossing over the anatomic course of the nervus occipitalis major and minor. When positioning of the needle is considered satisfactory, a standard 4-contact electrode is advanced through the needle, and the tip of the electrode is placed in close proximity of the nervus occipitalis major and minor. Correct position of the electrode is verified fluoroscopically and by intraoperative test stimulation of the awake patient. Once adequate coverage has been achieved, the electrode is sutured in place with nonabsorbable sutures using 2 plastic anchors provided by the manufacturer to minimize the possibility of movement. In case of a successful trial, defined as more than 50% of pain reduction, the patient has the second part of the surgical procedure performed under general anesthesia. The first incision is made along the retroauricular area in a vertical direction. The soft tissues are dissected down to the fascia, and a small pocket is created

above the fascia. The temporary electrode is removed, and the standard 4-contact electrode is then positioned in the same anatomic location. Once the electrode is positioned in the right place, the needle is removed and the electrode is anchored to the occipital fascia with nonabsorbable sutures using a plastic anchor. Next, a subcutaneous pocket is created below the clavicle on the same side to accommodate the neurostimulator. Extension cables are advanced through a subcutaneous tunnel, establishing a connection between the electrode and neurostimulator. At the end, all incisions are closed, cleaned and covered with sterile dressings [40].

5.5 Summary

Occipital neuralgia is responsible for neck pain and headache. No absolute data are available about its prevalence and incidence. History, clinical examination, and a positive test block with local anesthetic can provide an indication for the diagnosis.
A single infiltration of the nervus occipitalis major with corticosteroids and local anesthetic is advised. If the symptoms are resistant to infiltration with local anesthetic and corticosteroids, PRF of the nervus occipitalis can be considered. If pain persists, PRF of the ganglion spinale (DRG) C2 or C3 can be considered in a clinical trial setting. Because of the cost and invasiveness, subcutaneous nerve stimulation is placed last in the treatment algorithm and should only be performed in experienced centers.

This review was initially based on practice guidelines written by Dutch and Flemish (Belgian) experts that are assembled in a handbook for the Dutch-speaking pain physicians. After translation, the manuscript was updated and edited in cooperation with U.S./International pain specialists.

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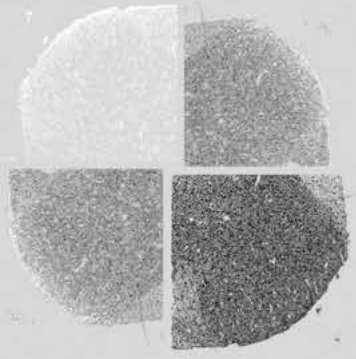

CHAPTER 6

Pulsed radiofrequency for the treatment of occipital neuralgia: a prospective study with 6 months follow-up

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Published in Regional Anesthesia and Pain Medicine, 2010 Mar-Apr;35(2):148-51

Abstract

Background and Objectives

Occipital neuralgia is a paroxysmal non-throbbing, stabbing pain in the area of the greater or lesser occipital nerve caused by irritation of these nerves. Although several therapies have been reported, no criterion standard has emerged. This study reports on the results of a prospective trial with 6 months of follow-up in which pulsed radiofrequency treatment of the greater and/or lesser occipital nerve was used to treat this neuralgia.

Methods

Patients presenting with clinical findings suggestive of occipital neuralgia and a positive test block of the occipital nerves with 2 ml of local anesthetic underwent a pulsed radiofrequency procedure of the culprit nerves. Mean scores for pain, quality of life and medication intake were measured 1, 2 and 6 months after the procedure. Pain was measured by visual analog and Likert scales, quality of life was measured by a modified brief pain questionnaire and medication intake was measured by a medication quantification scale.

Results

During a 29-month period, 19 patients were included in the study. Mean visual analog scale and median medication quantification scale scores declined by 3.6 units (P=0.002) and 8 units (P=0.006), respectively, after 6 months. Approximately 52.6% of patients reported a score of 6 (pain improved substantially) or higher on the Likert scale after 6 months. No complications were reported.

Conclusions

Pulsed radiofrequency treatment of the greater and/or lesser occipital nerve is a promising treatment of occipital neuralgia. This study warrants further placebo-controlled trials.

6.1 Introduction

The International Headache Society [1] defines occipital neuralgia (ON) as a paroxysmal non-throbbing, stabbing pain in the area of the greater or lesser occipital nerve. The pain is located in the suboccipital region, radiates over the scalp, and can be elicited by applying pressure over the course of the greater or lesser occipital nerve. Sometimes, hypoesthesia or dysesthesia is noticed. These findings, in combination with a short-term improvement after local anesthetic infiltration, establish the diagnosis [1]. In 85% of patients the ON is unilateral. The greater occipital nerve is more frequently involved (90%) compared to the lesser nerve (10%) [2]. No data are available about the incidence or prevalence. Irritation of the occipital nerves often lies at the origin of the neuralgia. Reasons for this irritation are diverse, ranging from vascular compression by aberrant vessels [3], giant cell arteritis [4], compression by osteogenic tumors [5], arthrosis [6], callus formation after vertebral fractures [7], schwannoma [8], C2 myelitis [9] or, as is most often the case, tendinomuscular compression [10]. Proposed treatments for ON include local injection therapy [11], subcutaneous electrical stimulation of the occipital nerves [12], acupuncture [13], neurolysis of the occipital nerves [14], dorsal root entry zone rhizotomy [15], or even ganglionectomy. However, there is no criterion standard for the treatment of ON. Pulsed radiofrequency (PRF) therapy is classified as a percutaneous minimal invasive treatment for pain. Descriptions about PRF therapy applied at peripheral nerves for the treatment of neuralgias are restricted to case reports precluding any conclusion to its effectiveness [16]. One case report is published about PRF therapy for the treatment of ON [17]. To date, no long-term prospective trials have been conducted concerning the treatment of ON. We present this first prospective clinical trial with 6 months of follow-up in which PRF current was used to treat patients with ON.

6.2 Materials and methods

6.2.1 Patient selection

The study was initiated following approval of our ethics committee (Commissie voor Medische Ethiek, Ziekenhuis Oost-Limburg, Genk, Belgium). Patients presenting with ON (for in- and exclusion criteria see Table 1) were included after obtaining their written informed consent.

Table 1. In- and exclusion criteria.

Inclusion Criteria:	Exclusion criteria:
Paroxysmal non-throbbing or lancinating pain in the area of the greater or lesser occipital nerve The pain starts suboccipitally and radiates over the vertex	Polyneuropathies whatever the etiology (diabetic, alcoholic, etc) Intracranial pathologic lesion Central neuropathic pain states
Hypoesthesia or dysesthesia in the area of the greater or lesser occipital nerve	Psychiatric comorbidity afflicting pain perception Previous interventions for treatment of the ON
Pressure over the course of the greater or lesser occipital nerve elicits the characteristic pain	Radiofrequency treatment of cervical facet joints Cervical epidural injections
Minimal score of 4 on the visual analog scale (VAS)	Fibromyalgia and chronic fatigue syndrome
50% or more reduction in the VAS after infiltration with local anesthetic	Neck surgery Coagulation disorders Infection at the infiltration sites Pregnancy Cervical spinal cord injury Intervertebral disc herniation at level C2-3 Neoplastic disease causing ON

An infiltration with local anesthetic (bupivacaïne 0.5%, 2ml per nerve) of the culprit occipital nerves was performed. Patients with 50% or more pain reduction on the visual analog scale (VAS) received a PRF treatment of the same occipital nerves. Correct injection was confirmed by the presence of scalp anesthesia in the dermatomes supplied by the lesser and/or greater occipital nerve. Infiltration sites for the greater and lesser occipital nerve were chosen according to the anatomic descriptions by Vital et al. [18], Becser et al. [19], Loukas et al. [20] and Natsis et al. [21]. More detailed information regarding the infiltration sites is provided in Figure 1.

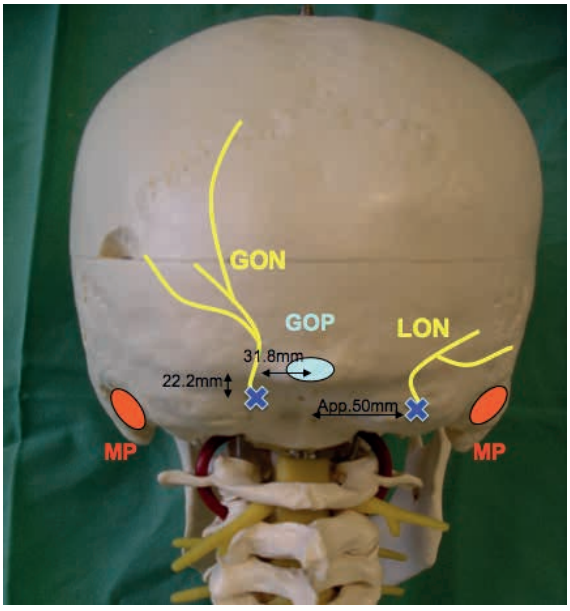

Figure 1. External anatomical landmarks for the infiltration of the greater and lesser occipital nerve. LON indicates lesser occipital nerve; GON, greater occipital nerve; GOP, greater occipital protuberance; MP, mastoid process; X, infiltration site; App, approximately.

6.2.2 PRF treatment of the occipital nerves

The target occipital nerves were identified using a 23-gauge CXE-6 needle (Cotop International BV, Amsterdam, The Netherlands, 60-mm/5-mm active tip) connected to a lesion generator (Cosman RFG-1B generator, Cosman medical Inc. Burlington, MA, USA) providing a 50-Hz, 0.5-V current. Correct needle position was confirmed by the presence of paresthesia in the concordant dermatomes with sensory thresholds lower than 0.5 V. PRF current consisted of 20-msec bursts with a frequency of 2 Hz and 45 V and was applied for 240 secs per nerve. Care was taken not to exceed 42° C. No local anesthetic was given in order to prevent unintentional anesthesia of the occipital nerves.

6.2.3 Measurements

The primary outcome measure of the study was the effect of PRF treatment on pain measured by a VAS and a 7-point Likert scale [22, 23]. Secondary outcome measures were improvement in quality of life and reduction in medication intake assessed by a modified brief pain questionnaire and a medication quantification scale (MQS) [24] (Grünenthal GmbH, Aachen, Germany), respectively. An example of the Likert scale, VAS, and modified brief pain questionnaire is provided below. VAS, modified brief pain questionnaire and MQS scores were obtained before infiltration and 1, 2 and 6 months after PRF treatment. The Likert scale was acquired 1, 2 and 6 months after PRF treatment. Inquiries about adverse effects were made at every visit.

6.2.4 Statistical analysis

StatPlus:mac version 2008 (AnalystSoft, Vancouver, Canada) software was used to analyze the data. Mean values of the VAS for pain and the modified brief pain questionnaire before and after PRF treatment were compared using a paired *t*-test at an α level of 0.05. One-tailed *t*-test probabilities reported with *P* values < 0.05 were considered statistically significant. Data of the MQS before and after PRF treatment were compared using a Wilcoxon matched pairs test. Data are presented as mean values ± standard error of mean (SEM) for the VAS for pain and the modified brief pain questionnaire and as median (range) for the MQS.

Likert Scale

7. The pain has disappeared.
6. The pain has improved substantially.
5. The pain has improved a bit.
4. The pain is the same.
3. The pain is a bit worse.
2. The pain is substantially worse.
1. The pain has become intolerable.

Modified Brief Pain Questionnaire

Mood

0 — No disturbance
10 — Highest imaginable disturbance

Daily activity

0 — No disturbance
10 — Highest imaginable disturbance

Sleep

0 — No disturbance
10 — Highest imaginable disturbance

Visual analogue scale

0 — No Pain
10 — Highest imaginable pain

6.3 Results

In a 29-month period (January 2006 to May 2008) 33 patients suspected with ON attended our multidisciplinary pain clinic. Thirteen patients had less than 50% improvement after infiltration of the occipital nerves with local anesthetic and were excluded from the study. Of the remaining 20 patients, 19 received PRF treatment. One patient refused PRF treatment because she was still without pain at the time the treatment was scheduled. Demographic data are presented in Table 2.

Table 2. Demographic data of the study population.

			Number of patients	Percentage	Mean age (years)	Range (years)
Gender	Female		15	75%	53.3	22-77
	Male		5	25%	57.6	43-72
Location	Left sided occipital neuralgia	GON	0	0%		
		LON	1	5%		
		GON + LON	8	40%		
	Right sided occipital neuralgia	GON	0	0%		
		LON	0	0%		
		GON + LON	2	10%		
	Bilateral occipital neuralgia	GON	0	0%		
		LON	0	0%		
		GON + LON	9	45%		
Total			20	100%		

GON: greater occipital nerve, LON: lesser occipital nerve

Mean VAS scores, mean modified brief pain questionnaire scores and median MQS scores of the 19 treated patients are presented in Figure 2. The mean±SEM VAS score before treatment was 7.5±0.4 and declined to 3.5±0.8, 3.5±0.7 and 3.9±0.8 at 1, 2 and 6 months, respectively (P<0.001, P<0.001, and P=0.002, respectively). The median MQS score before treatment was 11.2 (range 18.2) and declined to 4.4 (range 18.2), 3.4 (range 17.1) and 2.2 (range 17.1) at 1, 2, and 6 months, respectively (P<0.01, P=0.011, and P=0.006, respectively).

The results for the quality of life parameters (disturbance of daily activity, mood disturbance, and sleep disturbance) are presented in Table 3. Of the 19 patients 13 (68.4%), 11 (57.9%), and 10 (52.6%) mentioned a score of 6 or more on the Likert scale 1, 2, and 6 months after PRF treatment, respectively. No adverse effects were reported. The VAS score of the patient who refused PRF treatment was 6 at baseline and 0, 2, and 7 at 1, 2, and 6 months after infiltration with local anesthetic, respectively.

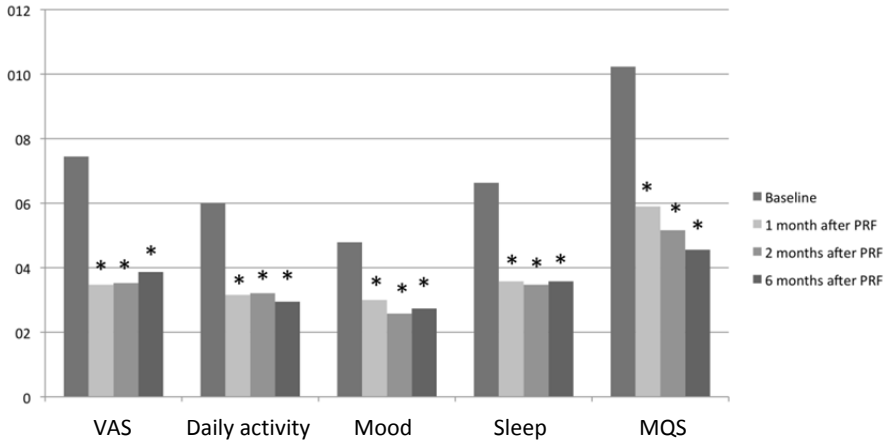

Figure 2. Mean scores for the visual analogue scale (VAS), disturbance of daily activity-mood-sleep and median score for the medication quantification scale at 1, 2, and 6 months after therapy. * $P<0.05$.

Table 3. Evolution of quality of life parameters 1, 2, and 6 months after treatment

	Baseline	1 mo	2 mo	6mo
Disturbance of daily activity	6±0.6	3.2±0.8 (*P*=0.035)	3.2±0.8 (*P*=0.004)	3±0.8 (*P*=0.002)
Mood disturbance	4.8±0.8	3±0.8 (*P*=0.06)	2.6±0.8 (*P*=0.025)	2.7±0.8 (*P*=0.037)
Sleep disturbance	6.6±0.8	3.6±0.9 (*P*=0.009)	3.5±0.9 (*P*=0.009)	3.6±1 (*P*=0.01)

Values are mean±SEM.

6.4 Discussion

This first prospective study on PRF for the treatment of ON shows a significant pain relief and reduced pain medication intake for 6 months. Sleep, mood and daily activity also improved during the 6 months of follow-up.

Kuhn et al. [25] prospectively identified 12 patients in the emergency department who met the International Headache Society criteria for ON. Following a positive test block (1-3ml of bupivacaine 0.5% with epinephrine), the patients received an infiltration with corticosteroids. In 70% of patients the pain recurred within 2 weeks. Prolonged pain relief for more than 2 months was observed in 20% of the cases. Comparable outcomes were found in a study performed by Hammond and Danta [2]. In our experiment only 1 patient (5%) had pain relief for more than 2 months after a single injection with local anesthetic. Mechanisms responsible for long-term pain relief after a single dose of local anesthetic still need to be elucidated. Injections with Botulinum toxin type A [26] in 6 patients relieved the sharp, shooting pain associated with ON, yet had no effect on the dull, aching pain. Quality of life measures exhibited some im-

provement. No significant reduction in pain medication was demonstrated. However, in a retrospective case series of 6 patients with occipital neuralgia Kapural et al. [11] were able to demonstrate a pain reduction after injection of Botulinum toxin A (VAS declined from 8±1.8 to 2±2.7) with a mean duration of 16.3±3.2 weeks. After implantation of a subcutaneous electrode at the level of C1 [27], 7 of 14 (50%) ON patients had significant to complete pain reduction. Of 10 patients, 3 had their neurostimulator removed (1 owing to infection, 1 owing to lead migration and spasm of the neck, 1 owing to spontaneous improvement of pain). In a case series of 6 patients with ON who underwent occipital nerve electrical stimulation, a reduction in VAS scores for pain (8.66±1.0 to 2.5±1.3) and pain disability index were noted after 3 months follow-up [12]. After performing intradural dorsal rhizotomies of C1 to C4 in 9 patients, Horowitz and Yonas [28] found prolonged complete relief in 44% of patients. Two patients (22%) had complications (wound infection, muscular neck pain resulting in reduced range of motion).

Compared with the aforementioned results of other treatment modalities, PRF treatment for ON shows a higher efficacy without any complications. Nevertheless, it should be mentioned that in 2 studies [27, 28] the follow-up was longer than in our study. No adverse effects were reported by the patients in our study.

Several limitations in our study are worth mentioning. First, although the VAS, Likert scale and modified brief pain questionnaires are validated tools in the quantification of pain, they are subjective outcome measures because they are dependent on personal interpretation and variations. With the use of the MQS, we tried to circumvent part of this subjective interpretation. Second, the small sample size limits the power of the outcomes. Finally, it is worth mentioning that uncontrolled trials have a tendency to overestimate treatment effects.

In conclusion, PRF for the treatment of ON shows promising results that need to be confirmed by larger placebo-controlled studies.

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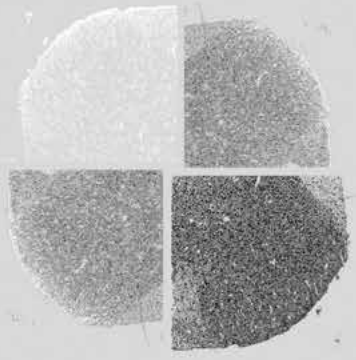

CHAPTER 7

Biological indicators for neuropathic pain in patients with chronic lumbar radicular pain due to failed back surgery syndrome

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Submitted

Abstract

Background

At present, development of biomarkers to detect the intensity or duration of pain has been unsuccessful. Instead, biomarkers to distinguish different types of pain are well within reach. Despite numerous immunological and neurochemical pathways involved in the generation of neuropathic pain have been discovered through animal research, physicians still rely on questionnaires to detect neuropathic pain in man.

Methods

We determined serum, plasma and CSF concentrations of TNF-α, BDNF, CGRP-α, NPY and GABA in patients with failed back surgery syndrome and neuropathic pain (NP-group) before and 4 weeks after spinal cord stimulation (SCS), and compared these values to serum and plasma concentrations of pain-free controls (PF-controls) and CSF concentrations of controls with nociceptive pain (NC-controls).

Results

BDNF and CGRP-α serum concentrations in the NP-group were markedly lower than in the PF-group ($P \leq 0.01$ and $P \leq 0.0001$, respectively) and rose after SCS ($P \leq 0.05$ in both cases), whereas initial serum and plasma concentrations of TNF-α ($P \leq 0.001$ and $P \leq 0.002$, respectively) and NPY ($P \leq 0.02$ and $P \leq 0.001$, respectively) and plasma concentrations of GABA ($P \leq 0.005$) were higher in the NP-group but unchanged after SCS. TNF-α was the only biomarker in CSF that was higher in the NP-group compared to the NC-group.

Conclusions

We present a series of changes in biomarker concentrations in blood and CSF that favor inflammation and jeopardize neuroprotection in failed back surgery syndrome and can be used as a biomarker set to distinguish neuropathic pain from other types of pain.

7.1 Introduction

The concept of biomarkers for pain is controversial because the expression of pain not only represents a nociceptive mechanism but also the processing of this nociceptive information at different spinal and supraspinal levels, not in the least by emotions and affective state. As such, a biomarker for pain itself can only be a surrogate for the mechanisms mentioned above. This said, biomarkers for the detection of the intensity or duration of pain seem unfeasible at present [1]. Instead, biomarkers to distinguish different types of pain are well within reach because recent investigations uncovered biomarkers that allowed for differentiation between primary headache disorders [2]. Along these lines, animal research has revealed a host of immunological and neurochemical pathways that are potentially involved in the initiation and maintenance of neuropathic pain (NP) [3-6]. To date, no application exists for these substances as biomarkers to distinguish NP from other types of pain in humans, and physicians still rely on clinical examination and questionnaires as screening tools to detect NP [7-9]. Clinical practice would strongly benefit from diagnostic tests based on the measurement of factors in blood and/or cerebrospinal fluid (CSF), because they would supplement the current subjective assessment of NP and improve research and treatment of this condition.

Among the various types of NP in humans, neuropathic low back and radicular pain are the most prevalent [10-12]. Failed back surgery syndrome (FBSS) constitutes a major source of this neuropathic low back and radicular pain. With a prevalence of 0.6% [13] and reports of up to 50% of lumbar surgeries resulting in FBSS [14], the disorder forms a heavy burden for patients and society. Drugs effective in diabetic polyneuropathy and postherpetic neuralgia fail to demonstrate superiority over placebo in radicular NP [15-17]. However, in a recent prospective randomized controlled multicenter trial, spinal cord stimulation (SCS) was superior to conventional medical management in successfully treating patients with FBSS [18]. Nevertheless, the exact mechanism of action by which SCS acts on NP is largely unknown.

In the present study we measured CSF and blood concentrations of a series of molecules involved in the pathophysiological processes of NP and investigated their possible usefulness as biomarker to distinguish NP from nociceptive pain in humans. Since in recent years the neuroinflammatory cascade, peptides of the calcitonin family, neurotrophins, and excitatory as well as inhibitory neurotransmitters have been implicated in the pathophysiology of NP [3, 4, 6, 19, 20], we focused on one well-established representative of each of these categories: tumor necrosis factor-α (TNF-α), calcitonin gene-related peptide-α (CGRP-α), brain-derived neurotrophic factor (BDNF), neuropeptide Y (NPY) and γ-aminobutyric acid (GABA). Their concentrations in blood and CSF of patients with NP have been compared with those in CSF of patients suffering from nociceptive pain and with those in the blood of pain-free controls. Furthermore, the effect(s) of SCS on these factors was studied by assessing their

concentrations in blood and CSF in patients with FBSS and NP, before and after 4 weeks of SCS.

7.2 Materials and methods

7.2.1 Study set-up

This single center study was approved by the Ethics Committee of our hospital (Commissie voor Medische Ethiek, Ziekenhuis Oost-Limburg, Genk, Belgium). Patients were recruited from our patient directory and from referrals to our tertiary pain clinic. Researchers obtaining patient questionnaires and performing serum, plasma and CSF analyses were unaware of the group the patient was allocated to. The timeline of the study is presented in Figure 1.

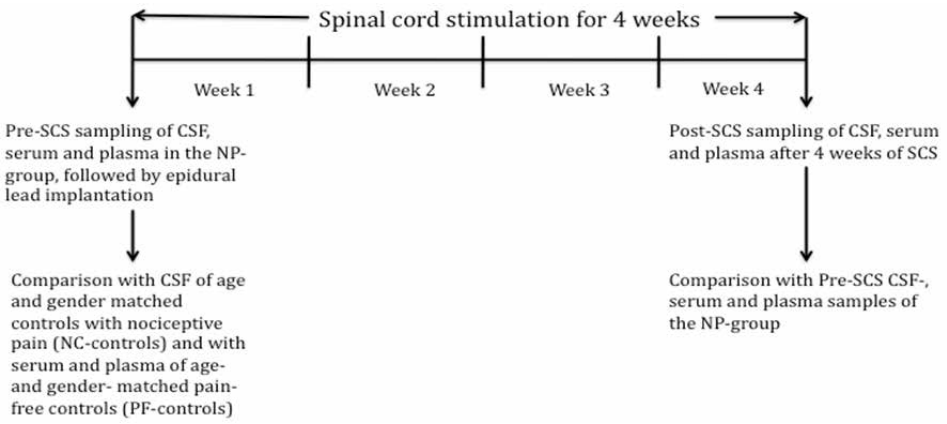


Figure 1. Timeline of the study.
Before (pre-) and after (post-) 4 weeks of spinal cord stimulation (SCS) cerebrospinal fluid (CSF), serum and plasma samples were taken from patients with neuropathic pain (NP). The pre-SCS serum and plasma samples were compared with age- and gender-matched controls with nociceptive pain (NC-controls) regarding CSF and pain-free controls (PF-controls) regarding serum and plasma. Post-SCS samples were compared with the pre-SCS samples.

In short, patients (NP-group) eligible for inclusion in the study underwent CSF, plasma and serum sampling before epidural lead insertion (pre-SCS). Next, an epidural lead was inserted and patients received SCS for 4 weeks. Thereafter, post-SCS samples from CSF, serum and plasma were taken again in the NP-group. The pre-SCS samples of the NP-group were compared to CSF samples of age- and gender-matched patients with nociceptive pain (NC-controls) and to serum and plasma samples of age- and gender-matched pain-free volunteers (PF-controls). Furthermore, the post-SCS samples of the NP-group were compared with the pre-SCS samples.

7.2.2 Patient population

Patients and controls were included after their written informed consent. Patients with NP (NP-group) suffered from FBSS and presented with predominant lumbosacral radicular pain, which was confirmed by a diagnostic nerve root block. Patients with nociceptive pain (NC-controls) were selected from patients undergoing lower limb surgery (total knee replacement, diagnostic knee arthroscopy) under spinal anesthesia. Pain-free controls (PF-controls) were healthy volunteers. NC- and PF-controls were age- and gender-matched to the NP-group. Before sample collection, NP-patients and NC-controls rated their pain score using a visual analogue scale (VAS) [21]. A validated Dutch DN4 questionnaire [22] was used to confirm the neuropathic nature of the pain (cut-off ≥ 4/10) in the NP-group and the non-neuropathic nature of the pain in the NC-controls (<4/10). Patients with conditions known to alter CSF, serum or plasma levels of the investigational substances were excluded from the study. In- and exclusion criteria are presented in Table 1. Depression and anxiety disorders were ruled out by the Montgomery-Asberg Depression Rating Scale (MADRS) from which the 4 somatic items were excluded to minimize the effect of the physical items when examining affective state (cut-off > 7) [23], and by psychiatric evaluation. Infection and coagulation disorders were ruled out by blood examination. Other exclusion criteria were eliminated by history taking and clinical examination.

Table 1. In- and exclusion criteria.

Inclusion criteria	Exclusion criteria
Neuropathic pain group:	Multiple sclerosis
Failed back surgery syndrome	Lupus erythematosus
Predominant radicular leg pain	Post-traumatic stress disorder
DN4 score ≥ 4	Depression
	Parkinson's disease
Nociceptive pain group	Acquired immunodeficiency syndrome
Pain in the leg requiring:	Stroke
- Total hip replacement	Head trauma or spinal cord damage
- Total knee replacement	Normal pressure hydrocephalus
- Diagnostic knee arthroscopy	Fibromyalgia or chronic fatigue syndrome
DN4 score < 4	Infections either locally or systemic
	Polyneuropathy (diabetic, alcoholic, drug-induced)
	Pregnancy
	Previous or current malignancies
	Inability to give informed consent
	Coagulation disorders
	Primary headache disorders

7.2.3 Cerebrospinal fluid sampling

All CSF samples were collected between 8 AM and 12 AM, to minimize possible effects of circadian rhythms. Lumbar puncture was performed at the L3-4, L4-5 or L5-S1 interspace depending on the location of previous lumbar surgery in the NP-group or the surgery the patient had to undergo in case of the NC-controls. Lumbar punctures were carried out by board-certified anesthesiologists and pain physicians familiar with the procedure. In short, the lumbar skin was disinfected with Hibitane Plus (chlorhexidin 0.5% in isopropanol 70%, Mölnlycke Health Care, Gothenburg, Sweden) and draped sterile. After local skin anesthetic (2 ml Linisol 2%, lidocaine 2%, Braun, Melsungen, Germany), a 120 mm 27G pencil point spinal needle (Braun, Melsungen, Germany) was introduced into the subdural space and 2 ml CSF was collected (2.5 ml syringe BD plastipak, Madrid, Spain). When the CSF was not visually clear, the patient was excluded from the study. CSF samples were stored at – 72° C until analysis.

7.2.4 Serum and plasma sampling

Blood samples were collected from a forearm vein between 8 AM and 12 AM to minimize possible effects of circadian rhythms (lithium-heparin tubes for plasma samples, silicone-silica SST II Advance tubes for serum samples and EDTA tubes for blood platelet count; Vacutainer, BD, Plymouth, United Kingdom). Serum samples were left to clot for 15 min to ensure platelet degranulation. Serum and plasma were obtained by centrifugation for 10 min at 4.500 rpm at room temperature (Eppendorf Centrifuge 5702R, Eppendorf, Hamburg, Germany). Supernatants were aliquoted and stored at -72°C until analysis. Blood platelet counts were determined by flowcytometry.

7.2.5 Measurements of BDNF, TNF-α, NPY, GABA and CGRP-α

CSF, serum and plasma concentrations were determined by commercially available high sensitivity enzyme-linked immunosorbent assays (ELISA) for BDNF (R&D systems Europe, Abingdon, United Kingdom; detection range, DR, was 20-4,000 pg/ml), TNF-α (Abazyme, Needham, USA; DR: 2.4-500 pg/ml), NPY (Peninsula Laboratories, Bachem, United Kingdom; DR: 10–39 pg/ml), CGRP-α (Nuclilab, Ede, The Netherlands; DR: 2.15-300 pg/ml) and GABA (ImmunDiagnostik AG, Bensheim, Germany; DR: 0.05-2 µmol/l) using the procedure recommended by the manufacturers.

7.2.6 Epidural lead implantation and spinal cord stimulation

Patients received epidural lead implantation under local anesthesia with light sedation (diazepam 5mg intravenously; Roche, Brussels, Belgium) and prophylactic antibiotics

(cefazoline 2g; Sandoz, Kundl, Austria). The lumbar skin was disinfected with Hibitane Plus (0.5% chlorhexidin in 70% isopropanol, Mölnlycke Health Care) and draped sterile. After local skin anesthetic with lidocaine (Xylocaine 2% with epinephrine 1:80.000, AstraZeneca, Amsterdam, The Netherlands) a Tuohy-needle was inserted into the T12-L1 epidural space via a paramedial approach and a percutaneous quadripolar epidural lead (Pisces quad compact model; Medtronic, Minneapolis, MN, USA) or a percutaneous octapolar epidural lead (Octad standard model; Medtronic) was advanced until full coverage of the painful dermatomes with paresthesia was attained. Next, the epidural lead was tunneled subcutaneously towards the lateral side and connected to an external trialing neurostimulator (Medtronic) for 4 weeks. Spinal cord stimulation parameters of the NP-group are presented in Table 2.

Table 2. Spinal cord stimulation parameters of patients in the neuropathic pain group. Data concerning voltage, pulse width and frequency are missing for patient 19. D indicates dorsal vertebra level.

Patient	Voltage (V)	Pulse width (msec)	Frequency (Hz)	quadri-/octapolar lead	position of the tip of the electrode
1	2.7	330	60	8	D7
2	3.4	330	60	4	D8
3	7.5	330	70	4	D9
4	5	420	60	4	D9
5	7.2	420	60	4	D9
6	1.7	330	60	8	D8
7	3.2	420	60	4	D9
8	2.5	450	60	4	D8
9	8	420	60	4	D9
10	3.7	330	40	8	D7
11	6	330	60	4	D8
12	4.5	420	60	4	D9
13	5.2	420	40	4	D10
14	5	420	60	4	D10
15	4	330	60	4	D9
16	5.5	450	60	4	D9
17	3	420	60	4	D10
18	2.4	360	60	4	D8
19	-	-	-	4	D11
20	4	300	60	4	D8

7.2.7 Statistical analyses

Statistical analyses were carried out using Prism 5.0d (GraphPad Software, San Diego, CA, USA). Because data were not normally distributed (Shapiro-Wilk normality test), non-parametric tests were used for analysis. A Mann Whithey-U test and Wilcoxon

matched-pairs signed rank test (confidence intervals 95%) were used to compare non-matched and age- and gender-matched data between groups, respectively. Spearman's correlation test (confidence interval 95%) was performed to investigate a possible correlation between serum, plasma, and CSF-concentrations. Data are presented as means ± SEM. α=5%.

7.3 Results

7.3.1 Patient and control characteristics

Over a period of 36 months (February 2009 - February 2012) 26 patients were screened for inclusion in the NP-group. Six patients were excluded due to a MADRS score >7. Of the remaining 20 patients, 12 were matched to NC-controls and 13 to PF-controls. The demographic data of the different groups are presented in Table 3.

Table 3. Patient and control demographics.

	Total NP-group (n=20)	NP-group matched to NC-controls	Matched NC-controls (n=12)
Male/female (%)	45/55	60/40	60/40
Age (years)	52±3	56±5	56±5
Etiology of pain	L4 radicular pain: 5% L5 radicular pain: 35% S1 radicular pain: 35% L5 & S1 radicular pain: 25%	L4 radicular pain: 8.3% L5 radicular pain: 25% S1 radicular pain: 41.7% L5 & S1 radicular pain: 25%	Meniscal tear: 70% Gonarthrosis 30%
VAS	85±2	78±5	77±6
Duration of pain (months)	48±6	45±8	33±14
Time (months) between diagnostic nerve root block and CSF collection	18±5		
Time (months) between surgery and sample collection	57±8		
		NP-group matched to PF-controls	Matched PF-controls (n=13)
Male/female (%)		44.5/55.5	44.5/55.5
Age (years)		47±3	47±3
Etiology of pain		L4 radicular pain: 0% L5 radicular pain: 38.5% S1 radicular pain: 38.5% L5 & S1 radicular pain: 23%	- - - -
VAS		85±2	-
Duration of pain (months)		53±8	-

L5 and S1 radicular pain was the most common etiology of pain in the NP-group, whereas in the NC-group this was a meniscal tear. There were no differences in the duration of pain nor in VAS-scores between the age- and gender-matched NP-group and NC-controls (45±8 months *vs.* 33±14 months, and 78±5 *vs.* 77±6, respectively). In the NP-group, the time between surgery and sampling of blood and of CSF for the study was 57±8 months and the time between diagnostic nerve root block and sampling of blood and of CSF for the study was 18±5 months.

7.3.2 Serum, plasma and CSF concentrations of BDNF, TNF-α, CGRP-α, NPY and GABA in patients with NP and in controls

BDNF

The data are presented in Figure 2a. Whereas in plasma there was only a trend towards a lower BDNF-concentration in the NP-group compared with PF-controls (1.26±0.28 ng/ml *vs.* 1.63±0.11 ng/ml, P=0.06, respectively), in serum of the NP-group the BDNF-concentration was clearly lower than in the PF-controls (6.16±2.07 ng/ml *vs*. 16.85±2.26 ng/ml, $P \leq 0.01$, respectively). In the NP-group and PF-group there was an increase in serum BDNF-concentration compared with plasma ($P \leq 0.0005$ and $P \leq 0.001$, respectively). No difference was seen in the number of platelets between the NP-group and PF-controls (258.6±25.5*1000 platelets/µl *vs.* 237.0±8.2*1000 platelets/µl) but the calculated amount of BDNF released per platelet (serum BDNF concentration *minus* plasma BDNF concentration/number of platelets [24]) was lower in the NP-group than in the PF-controls (22.8±11.7 ag/platelet *vs.* 61.6±8.4 ag/platelet, $P \leq 0.02$, respectively). In CSF of the NP-group and NC-controls, BDNF concentrations were below the detection limit (20 pg/ml) of the ELISA.

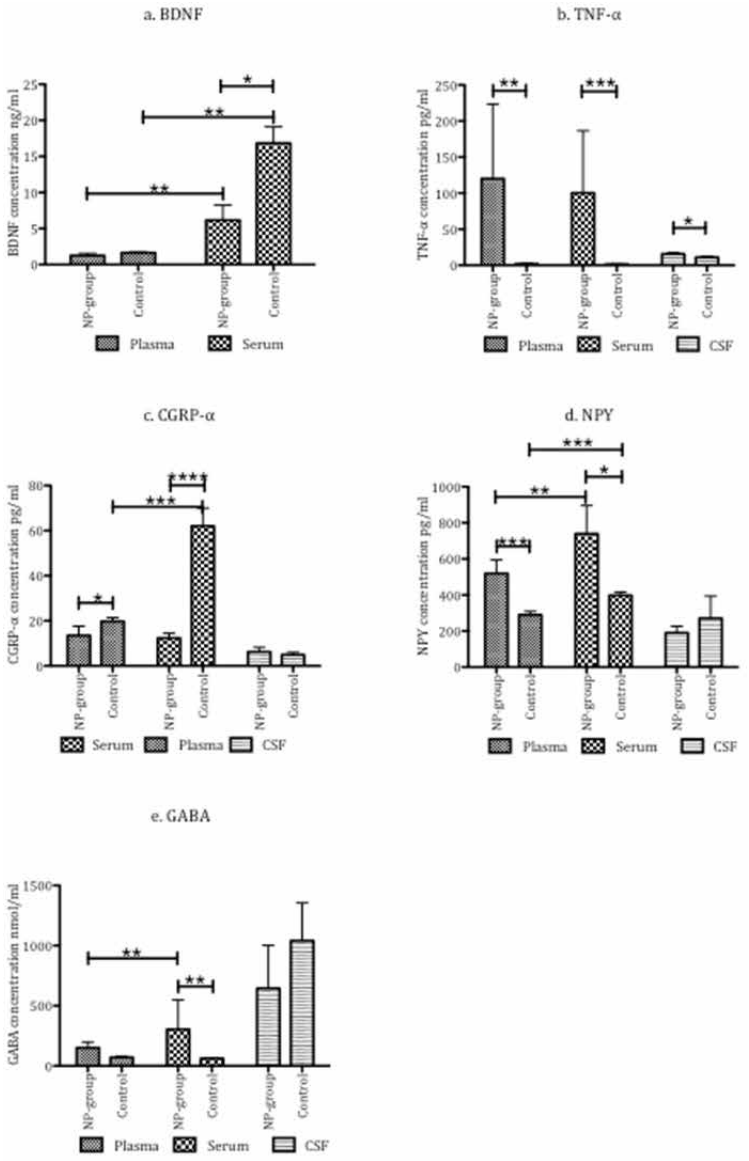
TNF-α

The data are presented in Figure 2b. TNF-α concentrations were higher in serum (100.4±86.3 pg/ml vs. 1.5±0.2 pg/ml, $P \leq 0.001$), plasma (120.2±103.2 pg/ml *vs.* 2.0±0.6 pg/ml, $P \leq 0.002$) and CSF (15.4±2.1 pg/ml *vs.* 11.0±1.0 pg/ml, $P \leq 0.02$) of NP-patients than of PF- and NC-controls, respectively. In the NP-group, serum and plasma concentrations of TNF-α did not differ from each other. In the NP-group, a positive correlation existed between serum and plasma concentrations of TNF-α on one hand and CSF concentrations of TNF-α on the other (r=0.86, $P \leq 0.0001$ and r=0.77, $P \leq 0.0001$, respectively).

CGRP-α

The data are presented in Figure 2c. CGRP-α concentrations in plasma and serum of the NP-group were lower than of PF-controls (13.5±4.2 pg/ml *vs.* 19.8±1.7 pg/ml, $P \leq 0.02$, and 12.4±2.1 pg/ml *vs.* 62.0±8.0 pg/ml, $P \leq 0.0001$, respectively). CGRP-α concentrations were markedly higher in serum than in plasma in the PF-controls

(*P*≤0.001) but not in the NP-group indicating decreased release of CGRP-α from blood platelets in the NP-group. No differences were noted in CSF concentration of CGRP-α between the NP-patients and CSF-controls (6.2±2.0 pg/ml *vs.* 5.0±1.1 pg/ml). However, in the NP-group we found a positive correlation between CSF and serum concentrations of CGRP-α (*r*=0.69, *P*≤0.005).



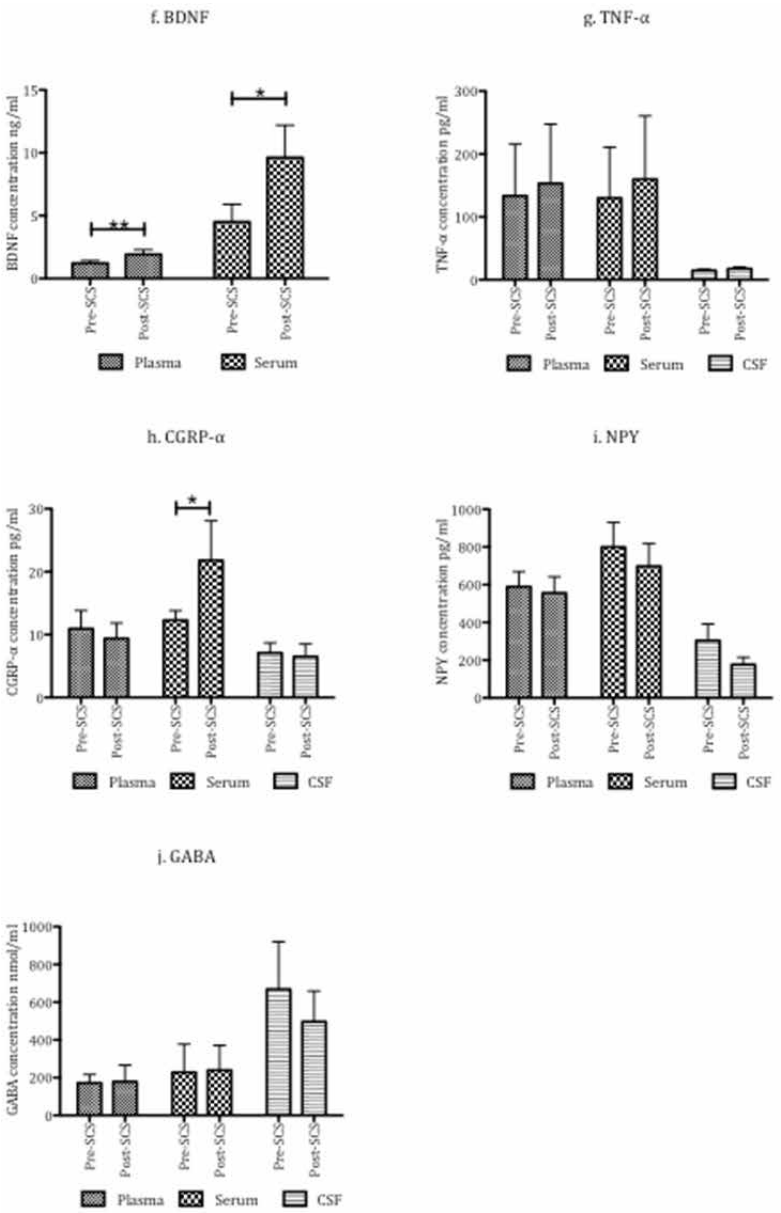


Figure 2. Serum, plasma and CSF concentrations of BDNF (a), TNF-α (b), CGRP-α (c) NPY (d) and GABA (e) in NP patients compared to controls and the effect of SCS on BDNF (f), TNF-α (g), CGRP-α (h), NPY (i) and GABA (j), concentrations in serum, plasma and CSF of NP patients. Capped lines delineate compared groups. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

NPY

The data are presented in Figure 2d. NPY-concentrations in plasma and serum of the NP-group were higher than in PF-controls (519.2±75.6 pg/ml *vs.* 289.2±21.0 pg/ml, $P \leq 0.001$ and 739.9±156.6 pg/ml *vs.* 397.7±18.0 pg/ml, $P \leq 0.02$, respectively). The NPY concentration in serum was markedly higher than in plasma, in both the NP-group and the PF-controls ($P \leq 0.005$ and $P \leq 0.001$, respectively) indicating substantial release of NPY from blood platelets. The calculated concentration of NPY in blood platelets of the NP-group was not different from that in PF-controls (781.6±409.7 zg/platelet *vs.* 468.4±69.37 zg/platelet). NPY-concentrations in CSF showed no difference between the NP-group and NC-controls (190.7±35.3 pg/ml *vs.* 270.6±123.0 pg/ml). In the NP-group, this concentration revealed positive correlations with the NPY-concentration in serum and plasma ($r=0.48$, $P \leq 0.02$ and $r=0.77$, $P \leq 0.0001$, respectively).

GABA

The data are presented in Figure 2e. Patients in the NP-group exhibited a higher plasma concentration of GABA than PF-controls (150.3±47.2 nmol/l *vs.* 70.1±7.7 nmol/l, $P \leq 0.005$) but no difference was observed between in GABA serum concentration (303.7±244.1 *vs.* 62.4±4.4 nmol/l). In the NP-group, the serum concentration of GABA was markedly higher than the plasma GABA concentration (227.9±149.8 nmol/l *vs.* 172.1±45.2, $P \leq 0.01$), whereas for the PF-controls only a trend for such a difference was seen (70.1±7.7 nmol/l *vs.* 62.4±4.4 nmol/l, $P=0.06$) suggesting release of GABA from blood platelets in patients with NP. We found no correlation between plasma and serum concentration of GABA on the one hand and CSF GABA concentration on the other. There was no difference in the GABA CSF concentration of NP-patients and NC-controls (644.3±358.1 nmol/l *vs.* 1040.0±315.4 nmol/l).

7.3.3 Effect of spinal cord stimulation on BDNF, TNF-α, CGRP-α, NPY and GABA concentrations in serum, plasma and CSF of NP patients

Four weeks treatment with SCS resulted in a clear reduction of VAS pain scores (85.6±2.3 *vs.* 37.8±5.4, $P \leq 0.0005$) in the NP-group, which was accompanied by a marked increase in serum and plasma BDNF concentrations (4.5±1.4 ng/ml *vs.* 9.6±2.6 ng/ml, $P \leq 0.05$ and 1.2±0.2 ng/ml *vs.* 1.9±0.4 ng/ml, $P \leq 0.01$, respectively; Figure 2f) and serum CGRP-α concentration (12.3±1.5 *vs.* 21.8±6.3 pg/ml, $P \leq 0.05$; Figure 2h).

However, serum and plasma concentrations of NPY, TNF-α and GABA showed no changes after 4 weeks treatment with SCS nor did the CSF concentration of any of the test substances (Table 4).

Table 4. Serum, plasma and CSF concentrations of tumor necrosis factor-α (TNF-α), calcitonin gene-related peptide-α (CGRP-α), neuropeptide Y (NPY) and γ-aminobutyric acid (GABA) in the NP-group before (Pre-SCS) and 4 weeks after spinal cord stimulation (Post-SCS).

	Serum			Plasma		
	BS	Post-SCS	P-value	BS	Post-SCS	P-value
NPY (pg/ml)	799.7±129.9	697.5±121.5	0.36	589.5±80.2	556.3±86.0	0.40
TNF-α (pg/ml)	129.9±81.1	159.9±100.8	0.41	133.3±82.2	153.0±94.32	0.31
GABA (nmol/l)	227.9±149.8	239.4±131.8	0.24	172.1±45.2	178.4±87.8	0.32
	CSF					
	BS	Post-SCS	P-value			
CGRP-α (pg/ml)	7.1±1.6	6.5±2.0	0.33			
NPY (pg/ml)	303.6±88.3	176.7±38.6	0.50			
TNF-α (pg/ml)	14.8±1.9	17.6±2.4	0.37			
GABA (nmol/l)	668.4±252.3	497.1±161.7	0.50			

7.3.4 Correlation of age, pain intensity and duration of pain with BDNF, TNF-α, CGRP-α and NPY concentrations

In the NP-group, we found no correlation between serum, plasma and CSF concentrations of BDNF, NPY, CGRP-α and TNF-α on the one hand and age, VAS-scores and duration of pain on the other.

7.4 Discussion

In the present study we investigated the usefulness of different substances to distinguish NP from other types of pain. The results show that patients with NP exhibit decreased serum concentrations of BDNF and CGRP-α and increased serum and plasma concentrations of TNF-α and NPY and increased plasma concentrations of GABA when compared to pain-free controls. Moreover, the CSF concentration of TNF-α was higher in patients suffering from NP than in patients with nociceptive pain. Four weeks treatment with SCS resulted in marked increases in serum and plasma BDNF concentrations and serum CGRP-α concentration.

To our knowledge, this is the first report about BDNF serum concentrations in humans with NP. Our observations are similar to the ones seen in neurodegenerative disorders such as multiple sclerosis, white matter atrophy, cognitive deficits and Alzheimer's disease [25-28], where pre-treatment BDNF concentrations are low and successful therapy restores BDNF levels [29]. Given the facts that 1) there is a bidirectional high-capacity saturable transport system for BDNF between the CNS and blood [30], 2) blood concentrations of BDNF reflect CNS levels across species [31] and 3)

BDNF promotes neuronal survival and proliferation [32], low serum BDNF concentrations may indicate a state of insufficient neuroprotection against nerve injury eventually leading to NP. Therefore, our data suggest that NP should be considered as a neurodegenerative disease. To find the origin of low serum BDNF concentrations in patients with NP, we determined the number of platelets in NP patients because platelets contain large stores of BDNF [33], but found no difference with PF-controls. However, when we calculated the amount of BDNF released per platelet it appeared that platelets collected from NP patients released 2.7-fold less BDNF than PF-controls. This finding indicates that NP patients either have an impaired release of BDNF from platelets and/or have depleted platelet BDNF-stores. Another possible cause of the lowered serum BDNF concentrations is the elevated TNF-α concentration in our NP-group because negative correlations between serum TNF-α and BDNF levels have been uncovered earlier [34]. Four weeks treatment with SCS resulted in a marked reduction of pain scores and in a marked increase in BDNF serum and plasma concentrations. It is unlikely that the increase in BDNF concentration results from the surgery to implant the SCS hardware because Chimienti et al. [35] showed a decrease in serum BDNF concentration after major abdominal surgery, which after 5 days subsequently returned to pre-surgery levels. Therefore, BDNF is a good candidate to be used as a biomarker to detect NP in FBSS and to monitor the effect of SCS treatment.

The elevated TNF-α concentrations in our NP patients are in contrast with earlier studies on complex regional pain syndrome and subacute sciatica, conditions associated with NP, that showed that in the CSF of patients levels of interleukin (IL)-1β, IL-6 and IL-8 were elevated but TNF-α had remained unaffected [36, 37]. However, in the study of Brisby and colleagues, patient selection was not made on the basis of neuropathic pain but by IASP-criteria for complex regional pain syndrome (CRPS) and clinical findings of sciatica and vertebral disc herniation [37]. Therefore, the biomarker profile these authors proposed could be specific for CRPS or subacute sciatica. Our findings are in line with results from research in patients with painful neuropathies showing elevated serum TNF-α concentrations in contrast with patients with painless neuropathies [38]. Also, in animal research using whole blood transcriptosomes the TNF-receptor superfamily gene was identified and validated as a biomarker for mechanical allodynia in a chronic constriction injury rat model [39]. Consequently, we suggest that TNF-α could be a possible biomarker for NP in FBSS.

CGRP-α concentration was decreased in our NP-group compared to PF-controls. Moreover, in the NP-group CGRP-α release from platelets was lower, closely resembling our observations concerning BDNF. These results indicate that storage and release of neurotrophins and neuropeptides from platelets may play a more important role in NP than currently supposed. A recent animal study [40] showed that CGRP-α has potent anti-inflammatory properties *in vivo,* mainly through inhibition of TNF-α release from macrophages and induction of IL-6 and IL-10. This means that the decreased plasma and serum levels of CGRP-α in our NP-group could be responsible for

the observed increases in TNF-α concentrations. Indeed, in patients afflicted by chronic CRPS, a condition associated with NP and pronounced local and systemic inflammation, the CGRP-α concentration was also lower than in controls [41]. However, the latter results were not replicated in a study on acute CRPS [42], suggesting a time-dependent factor in expression of biomarkers. The underlying mechanisms of lowered plasma and serum CGRP-α in patients suffering from NP were not investigated in our study and need further elucidation. Four weeks treatment with SCS also resulted in a marked increase in CGRP-α serum concentrations. Whereas this concentration can increase after soft tissue injury or hip fracture [43, 44] it seems unlikely such a rise would persist for 4 weeks after the precipitating incident. Moreover, the amount of tissue damage caused by implantation of a DCS system is minimal. Consequently, in addition to BDNF, CGRP-α seems to qualify for a biomarker to detect NP in patients with FBSS.

Our data reveal that NPY concentrations in plasma and serum of NP patients are higher than in PF-controls. Despite the fact that NPY is thought to be anti-nociceptive and neuroprotective [45], apparently, in our study these higher concentrations did not prevent the occurrence of NP. Our results are in line with two other studies, one in patients with sciatica [46] and another in patients with fibromyalgia [47], which failed to show a correlation between plasma NPY concentration and pain scores. In contrast, NPY concentrations are lowered in the affected limb of patients with CRPS. However, this could represent a reduction in sympathetic activity rather than a nociceptive effect because no comparison was made with a control group [48].

Although blood concentrations and platelet uptake of GABA have been extensively investigated in psychiatric disorders and epilepsy [49, 50] and GABA-ergic pathways play an important role in the development of NP [51], elevated plasma levels of GABA in NP patients are a new finding. Yet, previous data suggested that platelets can function as a peripheral model to study neuronal GABA-ergic functions [52, 53]. In animal research, results on the expression of GABA are highly dependent on the animal model used to induce NP and on the time point at which GABA concentrations are measured. However, a common denominator in animal models is the increased intracellular and decreased extracellular spinal concentration of GABA during the late phase of NP [19, 54]. Our results in humans show a trend towards a lower GABA concentration in the CSF of patients with NP, hence corroborating the results from animal research and implicating GABA-ergic pathways in the development of NP in humans with FBSS. However, the increase in extracellular GABA level as seen during SCS in animals could not be replicated in patients.

In our study, blood concentrations of TNF-α, NPY and CGRP-α correlated with CSF concentrations in NP patients. These findings are in line with animal studies demonstrating that the blood-spinal cord barrier (BSCB) after peripheral nerve injury becomes permeable to substances with a molecular weight up to 400 kDa [55]. This disruption of the BSCB may be triggered by pro-inflammatory mediators and gives rise to passive

influx of circulating cytokines and penetration of immune cells in the spinal cord. Similar findings were made in humans suffering from sciatica caused by disc herniation [56]. The fact that in CSF of NP patients only the TNF-α concentration markedly differed from that in NC-controls can be explained by the low concentration of these molecules in the CSF and, furthermore, from possible (small) differences between patients and controls on one hand and our small sample size on the other, which may have obscured the presence of small inter-individual differences.

We could not find a correlation between pain intensity or pain duration and the concentrations of the substances tested, indicating that these immunological and neurochemical factors should not be used to detect the amount of pain that patients experience, but rather be viewed as biomarkers to distinguish NP from other types of pain.

In conclusion this study shows a specific series of changes in the concentrations of immunological and neurochemical factors in blood and CSF that as a whole can be used as a biomarker set to distinguish NP from other types of pain in patients with FBSS (Figure 3). In blood these changes consist of elevated concentrations of TNF-α, GABA and NPY and decreased concentrations of BDNF and CGRP-α, consequently favoring inflammation and jeopardizing neuroprotection. In the CSF, elevated TNF-α concentrations have been detected. Moreover, BDNF and CGRP-α concentrations in the blood increase with successful SCS thereby allowing for monitoring effects of SCS.

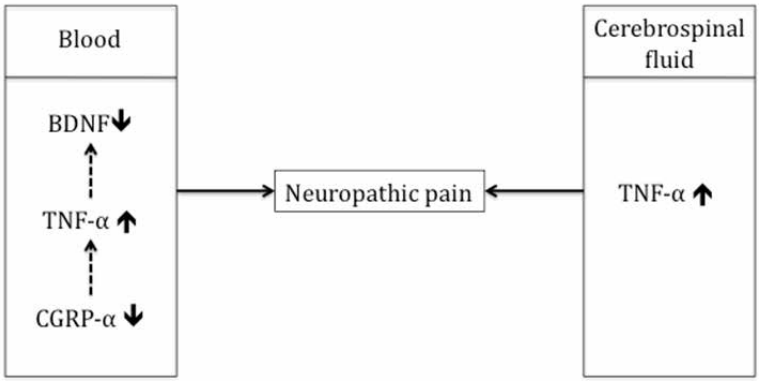


Figure 3. Blood and CSF concentrations of factors associated with NP. Dotted lines indicate assumed interactions.

7.5 References

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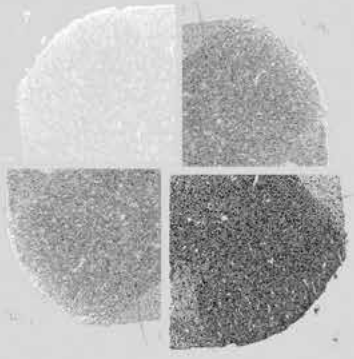

CHAPTER 8

General discussion and recommendations for future research

In this PhD thesis, possible new targets and treatments for neuropathic pain have been investigated. The research questions we addressed were:

- What is the role of BDNF in animals suffering from neuropathic pain?
- Do amitriptyline, gabapentin and minocycline reduce neuropathic pain behavior in a rat chronic constriction injury model via BDNF?
- Can minocycline and amitriptyline reduce lumbosacral radicular pain in humans?
- What is the current state of the art concerning occipital neuralgia?
- Can PRF at the level of the occipital nerves reduce neuropathic pain in occipital neuralgia?
- Can neurotransmitters and other neurochemical messengers be used as biological indicators for neuropathic pain?

Based on our investigations, we offer the following answers to these questions, and give recommendations for future research.

8.1 BDNF plays a pivotal role in the generation of neuropathic pain in animal models

In **Chapter 2**, we have assessed the role of BDNF in a number of well-studied animal models for neuropathic pain research by reviewing the literature, and we have concluded that different lesions give rise to different patterns of BDNF expression at the lesion site, in the DRG as well as in the dorsal horn [1]. Even when only one model is taken into account, the pattern of BDNF expression clearly depends on the amount of damage evoked. The mechanisms by which BDNF generates neuropathic pain are various but they all lead to a disinhibition or hyperexcitability of the dorsal horn, thereby stimulating nociceptive output from the spinal sensory system. However, the common denominator in every model is the fact that BDNF-antagonism, directly by scavenging BDNF molecules or reducing BDNF expression or indirectly by TrkB-receptor antagonism or second messenger inhibition, results in reduced neuropathic pain behavior [2-4]. Meanwhile, there is some inconsistency on this issue in the literature. Conflicting evidence, which suggests an anti-nociceptive role of BDNF, may be the result of interspecies differences in the expression of neuropathic pain and of BDNF [5]. Furthermore, inconsistency may be caused by intraspecies disparity between male and female animals in the expression of BDNF and neuropathic pain. Finally, BDNF has a clear dose-dependent effect on neuropathic pain: whereas low doses of exogenously administered BDNF can suppress mechanical allodynia, high doses have the opposite effect [6].

8.2 BDNF expression is not inhibited by current treatments of neuropathic pain, and therefore BDNF represents a new target in the treatment of neuropathic pain in humans

Current treatment of neuropathic pain aims at increasing central nervous system serotonin and norepinephrine levels by administering tricyclic antidepressants such as amitriptyline and selective norepinephrine reuptake inhibitors or at decreasing glutamate and substance P release by blocking the α_2-δ subunit of voltage-dependent calcium channels with anticonvulsant drugs such as gabapentin.

In **Chapter 3**, we have demonstrated that minocycline and amitriptyline but not gabapentin reduce neuropathic pain behavior in a rodent model of neuropathic pain when administered post-injury. Moreover, minocycline appeared to be the only drug that reduced spinal expression of BDNF. Figure 1 provides an overview of mechanisms implicated in the generation of neuropathic pain that are influenced by minocycline in the dorsal horn. From this figure it becomes clear that multiple pain signals converge on microglia while, in turn, the output signal from this microglia is rather limited, with BDNF as the most important downstream effector. Minocycline exerts several inhibitory actions on multiple pathways in spinal microglia and dorsal horn neurons, thereby causing reduced BDNF expression on one hand and attenuation of downstream effects of BDNF on the other. Consequently, it can be concluded that minocycline meets the criteria needed for a drug to be used in the treatment of neuropathic pain in humans. There is, however, a therapeutic window wherein minocycline should be administered post-injury in order to be effective [7, 8].

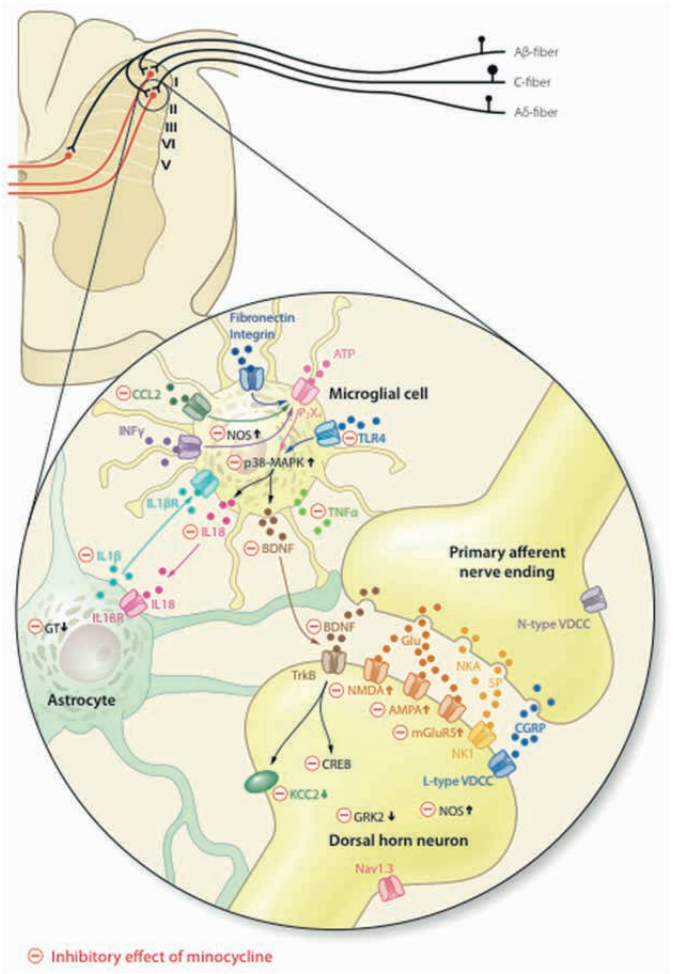

Figure 1. The inhibitory effects of minocycline on different pathways contributing to neuropathic pain. NOS: nitric oxide synthase; TLR4: toll-like receptor 4; CCL2: CC chemokine ligand 2; GRK2: G protein-coupled receptor kinase 2; CREB: cyclic AMP response element binding protein; IL18: interleukin 18; IL1β: interleukin 1β; p38-MAPK: p38 mitogen-activated protein kinase; P_2X_4: purinergic receptor; KCC2: potassium chloride exporter 2; TrkB: tyrosine kinase receptor B; mGluR5: metabotrope glutamate receptor 5; ATP: adenosine-5'-triphosphate; VDCC: voltage-dependent calcium channel; TNFα: tumor necrosis factor α, INFγ: interferon γ; Glu: glutamate; SP: substance P; CGRP: calcitonin gene-related peptide; NMDA: N-methyl-D-aspartate receptor; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NK1: neurokinin receptor 1; NKA: neurokinin A; Nav 1.3: voltage dependent sodium channel 1.3. *Illustration: Rogier Trompert, Medical Art.*

8.3 Minocycline is a therapeutic agent for lumbosacral radicular neuropathic pain in humans

The first animal study concerning minocycline and neuropathic pain treatment was published in 2003 [9]. However, only recently also human studies were undertaken to investigate this drug's applicability in this condition. Conversely, the use of amitriptyline [10] and gabapentin [11] in neuropathic pain treatment was first reported in humans with publications of animal studies exploring their molecular functioning following 26 years [12] and 1 year [13] later, respectively.

In **Chapter 4** we present evidence that minocycline provides short-term improvement of subacute lumbosacral radicular neuropathic pain with limited side-effects. Similar findings were noted in patients suffering from diabetic polyneuropathy [14]. Although a small randomized clinical trial (RCT) with the specific p38 MAPK inhibitor dilmapimod [15] showed promising results in patients with nerve injury, the results could not be repeated in a larger RCT with a similar compound (losmapimod) [16]. A chemokine receptor 2 antagonist, equally failed to show superiority over placebo in patients with posttraumatic neuralgia [17]. Therefore, p38 MAPK- and CCL2-inhibition are not the only mechanisms by which minocycline attenuates neuropathic pain in humans. The simultaneous action of minocycline on multiple intracellular pathways leading to reduced BDNF expression seems to determine the global clinical outcome, rather than a specific action of BDNF on a single enzyme or compound. Moreover, animal studies have revealed that the release of BDNF is biphasic. Initially, BDNF release takes place from a pre-existing cytoplasmic pool, after which newly synthesized BDNF is released via p38 MAPK- or CCR2/CCL2-dependent pathways [18]. Therefore, p38 MAPK- and CCR2/CCL2-inhibitors are unlikely to inhibit the initial phase of BDNF release and since BDNF is known to have an autocrine effect [19], p38 MAPK inhibitors may be unable to reverse the pathogenesis of neuropathic pain once this has started.

8.4 There are well-established diagnostic criteria for occipital neuralgia but no gold standard for its treatment

In **Chapter 5** the current literature concerning the epidemiology, diagnosis and therapy of occipital neuralgia has been reviewed. The International Headache Society defined occipital neuralgia as a paroxysmal shooting or stabbing pain in the dermatomes of the nervus occipitalis major or nervus occipitalis minor associated with hypo- or dysesthesia in the area of the nervus occipitalis major or minor as well as with tenderness to pressure over the course of the nervus occipitalis major or minor. This clinical presentation combined with a temporary improvement after a diagnostic block of the nervus occipitalis major and/or minor confirms the diagnosis [20]. Several therapies for

occipital neuralgia have been propagated such as infiltrations with corticosteroid or with Botulinum toxin A, PRF of the occipital nerves or the DRG of C2, and subcutaneous neurostimulation [21-25]. The quality of the evidence concerning the treatment of occipital neuralgia was graded by the system proposed by Guyatt et al. [26] and adapted by van Kleef et al. [27] which resulted in the following conclusions and recommendations:

- A single infiltration of the nervus occipitalis major with local anesthetic and corticoids may be considered for the treatment of occipital neuralgia.
- The results of infiltrations with Botulinum toxin A are contradictory.
- PRF of the occipital nerves should be considered if infiltration with local anesthetic and corticoids fail to provide sufficient pain relief.
- PRF of the DRG of C2 is only recommended in a clinical trial setting.
- Subcutaneous nerve stimulation can be considered in severe disabling pain that is unresponsive to other treatments.

In the absence of trials that compare these different treatments, it appears that there is no identifiable gold standard for the treatment of occipital neuralgia.

8.5 PRF of peripheral nerves is an effective treatment of neuropathic pain

In **Chapter 6** we demonstrate long-term pain relief of occipital neuralgia after PRF of the occipital nerves. Moreover, in a follow-up study we have built on these findings and discovered that PRF not only reduces pain intensity but also neuropathic symptoms. Eight weeks after PRF treatment of the occipital nerves, the VAS and DN4 scores were significantly reduced to baseline (7.6±0.4 *vs.* 4.5±0.6, $P \leq 0.001$ and 4.5±0.4 *vs.* 3.1±0.5, $P \leq 0.001$, respectively; P. Vanelderen, unpublished results). Recent investigations show that a traumatic inciting event, low diagnostic block volumes and employment of several bouts of PRF correlate with a positive outcome after PRF of the occipital nerves [28]. Also recently, the first randomized, double-blind, placebo-controlled trial involving PRF of the peripheral intercostal nerves was performed to treat neuropathic pain in patients with post-zoster neuralgia [29]. PRF was associated with lower pain scores and reduced need for analgesics 6 months after therapy. The mode of action of PRF still needs to be clarified. Whereas many investigations have been performed on the effects of PRF on the central nervous system, animal research data concerning PRF on peripheral nerves are sparse. After PRF, Erdine et al. [30] and Protasoni et al. [31] found an acute as well as a prolonged increase in smooth endoplasmic reticulum in DRG neurons with swollen cisternae and numerous vacuoles in ganglion cells. Furthermore, myelinated axons revealed pathological features such as aberrant myelin coverage. Also after PRF, pain processing neurons in the DRG showed upregulation of c-Fos [32, 33] and ATF-3 [34], markers for neuronal activity and cellular stress. All these data suggest that PRF applied at the level of the DRG induces stress or

injury in nociceptive neurons thereby leading to reduced synaptic transmission [35]. Spinal descending adrenergic and serotonergic pathways as well as the endogenous opioid system seem to play important roles in the analgesic action of PRF [36, 37]. In a rodent inflammatory pain model, PRF applied to the sciatic nerve was unable to attenuate mechanical hyperalgesia, as opposed to PRF administered to the DRG [38]. However, in a rodent model of neuropathic pain (spared nerve injury) PRF applied at the sciatic nerve reversed mechanical allodynia within 24 hours together with a down-regulation of TNF-α and IL-6 in the sciatic nerve and DRG and an up-regulation of the $GABA_B$-R1 and the 5-HT3 receptor as well as of Na^+/K^+-ATPase in the DRG [39]. We may conclude that in neuropathic pain states, PRF applied at the peripheral nerve and DRG reduces the expression of pro-inflammatory cytokines and blocks synaptic nociceptive transmission, similarly to what is seen with microglial inhibition and BDNF antagonism.

8.6 BDNF, CGRP-α, TNF-α and NPY may serve as biological indicators for neuropathic pain

In **Chapter 7** we have assessed the concentrations in the blood and in the CSF of key molecules known to participate in the generation and maintenance of neuropathic pain of patients suffering from chronic lumbosacral radicular neuropathic pain caused by failed back surgery syndrome. We found decreased serum concentrations of BDNF and CGRP-α and an increased concentration of TNF-α in the blood and CSF as well as an increased concentration of NPY in serum and plasma and an increased concentration of GABA in plasma. Moreover, successful treatment by spinal cord stimulation increased serum concentrations of BDNF and CGRP-α. The low serum BDNF concentrations in these patients may have resulted from impaired BDNF release from platelets and/or from depleted platelet BDNF-stores. We specifically searched for biomarkers to differentiate neuropathic pain from other types of pain. Since the expression of pain is not only the result of nociception but also of a myriad of biopsychosocial factors that influence pain perception, biomarkers will hardly ever help to predict the pain a patient will experience in an accurate quantitative way, but they can certainly help us to distinguish different types of pain thus allowing for better targeted therapies. Since neuropathic pain affects neurochemical messenger profiles that themselves change over time as well, we should consider multiple candidate substances at multiple time points during treatment.

8.7 Recommendations for future research

Up to now, relatively little attention has been paid to differences in neuropathic pain mechanisms in animal models with regard to species and gender. Also, animal studies

in neuropathic pain research often use a pre-emptive study design, a paradigm that hardly reflects the clinical setting where patients seek medical advice after pain has arisen. Therefore, we recommend that future research will take into account species and gender differences, and involves post-injury drug administration to better mimic the clinical situation.

Forthcoming research in which therapy of neuropathic pain with minocycline is investigated should use larger patient cohorts, different etiologies of neuropathic pain and longer follow-up periods to determine the decisive place of this drug in the treatment of neuropathic pain. Also, since lumbosacral radicular pain is far more prevalent than postherpetic neuropathic pain and diabetic polyneuropathy, research on the pharmacotherapy of this condition deserves increased attention.

There is a need for trials that compare the effects of PRF at the level of peripheral nerves with those of other treatments of peripheral neuropathic pain in order to establish good treatment algorithms for this condition. In view of the similarities between microglial inhibition and the effect of PRF, future studies concerning the mode of action of PRF should focus on the modulation of microglial activity and BDNF expression.

In future biomarker research there should be adequate matching of patients and controls because in healthy controls significant variations in the concentration of such substances have been demonstrated with regard to age, gender and body weight, and diurnal and seasonal variation [40-42].

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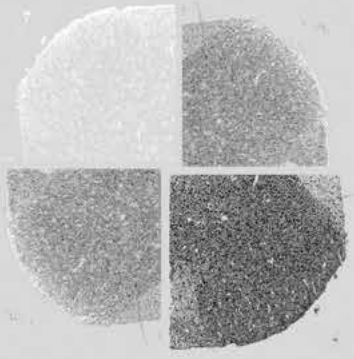


Summary

The aim of this thesis research was to investigate new targets and treatments for neuropathic pain.

In **Chapter 1** we provide a brief overview of the definition, epidemiology and pathophysiology of pain in general and neuropathic pain in particular. Next, the different neuropathic pain syndromes and their treatments that we studied in this thesis are discussed with a special emphasis on potentially new drugs.

In **Chapter 2** we summarize the role of the growth factor BDNF in four animal models of neuropathic pain (chronic constriction and transection of the sciatic and spinal nerves). BDNF is involved in the generation and maintenance of neuropathic pain in each of the above mentioned animal models. Moreover, inhibition of BDNF reduces neuropathic pain behavior in these animal models, thus making this growth factor a new target for the treatment of neuropathic pain.

The effect of amitriptyline and gabapentin, both first-line treatments for neuropathic pain, and minocycline, a tetracycline antibiotic drug and microglial inhibitor, in a rat chronic constriction injury of the sciatic nerve is presented in **Chapter 3**. Amitriptyline and minocycline both reduced neuropathic pain as opposed to gabapentin, which showed no effect. Moreover, minocycline was the only drug that reduced spinal BDNF expression. Therefore, minocycline can be a valuable new treatment for neuropathic pain in humans.

The findings of our animal study were translated into a clinical trial in **Chapter 4** by studying the effect of minocycline, amitriptyline and placebo in patients suffering from lumbosacral radicular pain. Both minocycline and amitriptyline reduced radicular pain compared to placebo. Moreover, patients treated with minocycline experienced less side-effects.

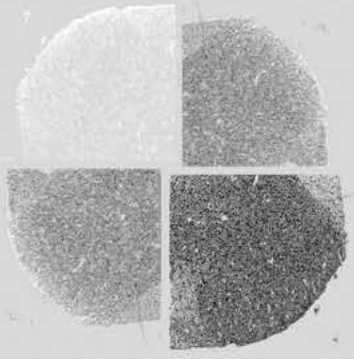
In **Chapter 5** we reviewed the literature concerning occipital neuralgia and proposed evidence-based treatment guidelines according to the current state of the art.

The long-term effect of PRF on occipital neuralgia was investigated in **Chapter 6**. PRF significantly reduced pain intensity and the need for analgesics, while improving quality of life of patients.

The usefulness as a biological indicator of several neurochemical messengers involved in the generation and maintenance of neuropathic pain in patients suffering from chronic lumbosacral radicular pain was investigated in **Chapter 7**. We found decreased blood concentrations of BDNF, CGRP-α and increased concentrations of TNF-α in the blood and CSF as well as increased blood concentrations of NPY and GABA. Moreover,

successful treatment with spinal cord stimulation restored blood concentrations of BDNF and CGRP-α.

In **Chapter 8** we discussed the results of the thesis research in a broad and integrated context and made recommendations for future research into the origin(s) and therapy of neuropathic pain in animals and humans.



Nederlandse samenvatting

Het doel van dit promotieonderzoek was om nieuwe aangrijpingspunten en behandelingen van neuropathische pijn te vinden.

Hoofdstuk 1 behandelt op beknopte wijze de definitie, epidemiologie, pathogenese en pathofysiologie van pijn in het algemeen en van neuropathische pijn in het bijzonder. Voorts worden de verschillende neuropathische aandoeningen en hun behandelingen beschreven die in dit onderzoek aan de orde zijn gekomen, met bijzondere aandacht voor aangrijpingspunten voor potentiële nieuwe geneesmiddelen.

De rol van de groeifactor BDNF in vier verschillende proefdiermodellen van neuropathische pijn (chronische constrictie *c.q.* doorsnijding van de nervus ischiadicus of van spinale zenuwen) wordt beschreven in **Hoofdstuk 2**. BDNF blijkt betrokken te zijn bij het ontstaan en aanhouden van neuropathische pijn in ieder beschreven diermodel. Bovendien neemt het neuropathische pijngedrag bij de proefdieren af wanneer de neuronale afgifte van BDNF wordt geremd. Hierdoor vormt BDNF een nieuw aangrijpingspunt voor de behandeling van neuropathische pijn.

In een chronisch constrictie model van de n. ischiadicus van de rat zijn de antineuropathische effecten van het tetracycline antibioticum en remmer van microglia, minocycline, vergeleken met die van twee huidige geneesmiddelen tegen neuropathische pijn, amitriptyline en gabapentine (**Hoofdstuk 3**). Zowel amitriptyline als minocycline verminderden het neuropathische pijngedrag bij de proefdieren. Dit was in tegenstelling tot gabapentine, dat geen effect op dat pijngedrag vertoonde. Bovendien bleek alleen minocycline in staat om de biosynthese van BDNF in het ruggenmerg te onderdrukken. Daarom kan minocycline beschouwd worden als een veelbelovend nieuw geneesmiddel voor de behandeling van neuropathische pijn bij mensen.

In **Hoofdstuk 4** wordt beschreven hoe de resultaten van het bovengenoemde dieronderzoek zijn toegepast in een klinische studie waarbij patiënten die lijden aan lumbosacrale radiculaire pijn behandeld werden met amitriptyline, minocycline of een placebo. Zowel minocycline als amitriptyline verminderden de intensiteit van de neuropathische pijn, maar patiënten die behandeld waren met minocycline ondervonden minder bijwerkingen.

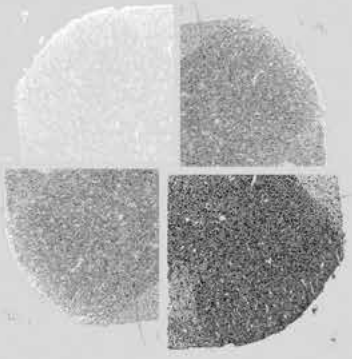
Hoofdstuk 5 betreft een literatuurstudie naar de behandeling van occipitalis neuralgie. Op basis hiervan zijn een aantal richtlijnen geformuleerd voor de behandeling van deze aandoening.

In **Hoofdstuk 6** wordt aangetoond dat gepulseerde radiofrequente therapie van de occipitale zenuwen leidt tot een daling van de pijnintensiteit en het medicijngebruik bij

patiënten die lijden aan occipitalis neuralgie. Bovendien trad er een belangrijke verbetering van de levenskwaliteit op.

Bij patiënten met chronische lumbosacrale radiculaire pijn is van een aantal belangrijke zenuwcelmoleculen nagegaan of zij gebruikt zouden kunnen worden als 'biomarker' voor pathofysiologische processen die betrokken zijn bij het ontstaan en/of aanhouden van neuropathische pijn (**Hoofdstuk 7)**. Bij deze patiënten werd een daling waargenomen van de concentraties van BDNF en het neuropeptide CGRP-α in het bloed en een stijging van de concentratie van de tumor necrosefactor TNF-α in het bloed en in het cerebrospinaal vocht evenals een stijging van de concentraties van het neuropeptide Y en de neurotransmitter GABA in het bloed. Door behandeling met dorsale streng stimulatie van het ruggenmerg konden de bloedconcentraties van BDNF en CGRP-α weer teruggebracht worden naar hun normale niveaus.

In **Hoofdstuk 8** zijn de resultaten van dit onderzoek in een brede context geplaatst en worden tot slot aanbevelingen gedaan voor verder onderzoek naar het ontstaan en de behandeling van neuropathische aandoeningen bij dier en mens.



Dankwoord

Door mijn drijvende krachten bij deze thesis, Prof. dr. Vissers, Prof. dr. Roubos, Prof. dr. Kozicz en dr. Van Zundert, kan ik vandaag deze woorden in dit boek schrijven. Naast jullie immense kennis in jullie vakgebieden, heb ik gepoogd om een aantal kenmerken samen te vatten dewelke mij steeds zullen bijblijven.

Kris, bedankt om samen met mij dit thesistraject te starten en gelukkig ook te voltooien. Jouw optimisme en drijfkracht lijken onuitputbaar. Na ieder overleg in Nijmegen reed ik met een schare aan nieuwe ideeën en perspectieven terug naar België. En over rijden gesproken, ik heb een kleine rekensom gemaakt over het aantal kilometers dat deze thesis op de teller gezet heeft: 35.000km. Als dit getal onwaarschijnlijk lijkt, zal het volgende zowaar nog ongelofelijker klinken: slechts 2 verkeersboetes op al deze kilometers.

Eric en Tamás, plots zat daar een Belg in een Nederlandse aio-kamer, bedankt om mij met open armen te ontvangen. Eric, de snelheid en accuraatheid waarmee jij manuscripten kan doornemen zijn verbijsterend. Jouw kennis van de Engelse taal maakt mij nog steeds nederig. Tamás, dankzij jou kon ik een luik met immunocytochemische kleuringen toevoegen aan mijn thesis, de cover art op de omslag van het boekje is hier een mooie getuigenis van.
Jan, bovenop de steun bij het klinische werk, had jij de gave om de focus van mijn aandacht van een brandpunt naar een helikopterzicht te expanderen waardoor het onderzoek en de resultaten plots veel duidelijker bleken en alles in zijn context viel. Ik dank jullie allen voor de voorbije 7 jaar.

Een bijzonder woord van dank wens ik te richten aan de leden van de beoordelingscommissie: Prof. dr. Harry van Goor, Prof. dr. Maarten van Kleef en Prof. dr. Guillén S.E. Fernández-Reumann. Bedankt dat jullie, ondanks een overvolle agenda, toch de tijd vrij maakten om mijn manuscript kritisch door te lezen en goed te keuren.

Ik wil mijn paranimfen, Pieter en Kim, bedanken voor het opnemen van deze vaak ondergewaardeerde taak, bovenop hun drukke klinische activiteiten. Dankzij jullie zal deze dag vlot verlopen.

Beste collega's, stafleden van de Kritieke Diensten van het Ziekenhuis Oost-Limburg, hoewel enkel mijn naam op dit doctoraat prijkt, is dit nochtans het werk van ons allen. Door jullie toewijding bleef ik *up to date* over de laatste ontwikkelingen in de verschillende deeldomeinen van ons vakgebied: de laatste nieuwe behandelingen in zake intensieve zorgen en urgentiegeneeskunde, transoesofagale echocardiografie, echogeleide locoregionale anesthesie,… zodat ik mij ten volle kon richten op deze thesis. Dank aan dr. Maud Beran, dr. Willem Boer, dr. Lieven Buyse, dr. Micheline Cauberg, dr. Bernadette Claessens, Prof. dr. Cathy De Deyne, dr. Jacques Deghislage, Prof. dr. Raf De Jongh, dr. Dimitri Dylst, dr. Kim Engelen, dr. Tom Fret, dr. Gertie Goossens, dr. Christel Ivens, Prof. dr. Frank Jans, dr. Mouloud Kalaai, dr. Luc Merckx, dr. Johan Van

Canneyt, dr. Margot Vander Laenen, dr. Luc Van Keer, dr. Jeroen Van Melkebeek, dr. Sven Van Poucke en dr. Guy Vundelinckx voor jullie steun en interesse in mijn thesis. Dr. Heylen, beste René, als diensthoofd slaag je er telkens in te anticiperen op de noden van de dienst ver vooraleer deze boven water komen. Wetenschappelijk werk is steeds een van deze noden geweest en zoals vaak had je gelijk. Bedankt om deze thesis te steunen.

Mijn speciale dank gaat naar mijn collega's van het Multidisciplinair Pijncentrum: dr. Pieter De Vooght, dr. Roel Mestrum, dr. Martine Puylaert en dr. Jan Van Zundert. Dankzij jullie vorderde het klinische luik van mijn onderzoek met rasse schreden. Jullie interesse in de *'black art'* van het promoveren was een onmisbare steun.

Het dieronderzoek van mijn thesis was niet mogelijk geweest zonder de hulp van Tom Rouwette, Ilona van den Brink en Francien van de Pol. Tom, een aantal jaren na jou ben ik dan eindelijk aan mijn promotie toegekomen. Bedankt voor de leerrijke discussies en de fijne tijd dewelke wij doorbrachten in de aio-kamer. Ilona en Francien, jullie toewijding en kennis van het opereren, verzorgen en uitvoeren van metingen bij de proefdieren kent geen gelijke. Jullie waren allen van grote waarde bij mijn dieronderzoek. Een vaak vergeten dank richt ik aan mijn proefdieren dewelke hun leven gaven voor de wetenschap zodat wij er als mensen beter van worden. Tony Coenen, Frouwke Kuipers-Kwant en Elisabeth Pierson wil ik bedanken voor hun hulp bij de immunocytochemische kleuringen en de fluorescentiemicroscopie. Prof. dr. Joris Penders en Carmen Reynders waren van grote waarde bij het uitvoeren van de ELISA's in de research naar biomarkers.

Prof. Bert Joosten en dr. Fons Kessels wil ik bedanken voor hun wijze raad bij de celkleuringen en de statistiek, respectievelijk en om het manuscript van het artikel in Hoofdstuk 4 kritisch na te lezen.

Het 'MPC-team': onze secretaresses, kinesisten, psychologen, verpleegkundigen en study nurses verdienen mijn bijzondere waardering. Zij maken ons werk op de pijnkliniek en bijgevolg deze thesis mogelijk.

Een grote schare aan opleiders heeft mij gevormd tot de arts die ik vandaag ben en daarvoor wil hen bedanken.

In het AZ Sint-Elisabeth te Turnhout zette ik mijn eerste stappen in de wereld van de anesthesie. Dr. Maurits Soetens, dr. Marc De Vel, dr. Paul Van Der Aa, dr. Agnes Van der Donck, dr. Herman Meeuwis, dr. Francis Smolders, dr. Filiep Soetens en dr. Marc Vanhoof transformeerden een *wannabe* internist om tot een anesthesist. Bedankt voor mijn opleiding en de mogelijkheid om de TOEFL en de USMLE's af te leggen.

Nadien vervolledigde Prof. dr. Eugène Vandermeersch en zijn staf mij tot een volleerd anesthesist. In het bijzonder wil ik Prof. dr. Marc Van de Velde (obstetrische anesthesie) en dr. Marleen Verhaegen (B-kern, anesthesie voor abdominale en oncologische heelkunde), bij wie ik de meeste tijd van mijn universitaire opleiding doorbracht, bedanken voor de kennis dewelke ik bij hen mocht vergaren. In het UMC Maastricht, bij Prof. dr. Maarten van Kleef leerde ik neuromodulatie technieken, terwijl dr. Jaap Patijn mij wegwijs maakte in het klinisch onderzoek bij pijnpatiënten.

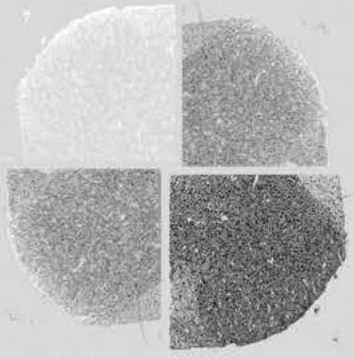
Dr. Jan-Willem Kallewaard, en dr. Michel Terheggen leerden mij de techniek van de epiduroscopie.

And last but not least, dr. Richard Derby of the Spinal Diagnostics and Treatment Center in Daly City, California, USA. Rick, thanks to you and your team for welcoming me as one of your own. You showed me the American way of performing pain therapy. I will never forget Saint Patrick's day (at least not the parts I can remember) in San Francisco.

Aan mijn ouders, grootouders en schoonouders: jullie geloofden meer in mij, dan ik in mijzelf. Bedankt voor de opofferingen en de mogelijkheden die ik kreeg. Ik hoop dat ik de verwachtingen ingelost heb.

Viktor, mijn zoon en bovenal mijn vriend. Zo veel van jou herken ik in mezelf maar iedere dag zie ik nog meer: de vechtlust, het ongeduldige maar voornamelijk de wilskracht om het beter te doen dan mij. De tijd is aangebroken om nog veel verder te zien.

Eva, mijn grootste schat, echtgenote en bovenal zielsverwante. Zonder jouw steun was deze thesis er nooit gekomen en was ons gezin niet wat het nu is. Hoe wij soms hetzelfde denken en zeggen grenst aan het griezelige. Al moest ik nog duizend levens leiden in duizend werelden, ik zou jou steeds terugvinden. Bedankt om mijn levensgezel te zijn in deze wereld.



Curriculum vitae

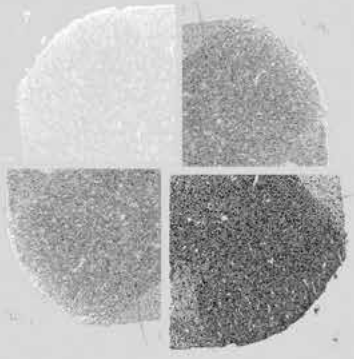
Pascal Vanelderen was born on April 1, 1974 in Sint-Truiden, Belgium. He finished high school at the Sint-Trudo Instituut, Sint-Truiden in 1992 and went on to study medicine at the Limburgs Universitair Centrum, Diepenbeek (renamed as University Hasselt) where he obtained the bachelor degree in medical sciences in 1995. The master degree was acquired *magna cum laude* at the Katholieke Universiteit Leuven in 1999. Initially, he started a residency in internal medicine (dr. Engelaar) in the Elisabeth Hospital in Turnhout, but he switched to a residency in anesthesiology (dr. M. Soetens) in the same hospital until 2003 and completed his residency in 2005 in the Universitair Ziekenhuis Gasthuisberg in Leuven (Prof. dr. E. Vandermeersch) and the Ziekenhuis Oost-Limburg in Genk (dr. R. Heylen). He is a staff member in the latter hospital in the department of anaesthesiology, intensive care medicine, emergency medicine and multidisciplinary pain therapy.

During his bachelor education Pascal followed additional courses in methodology in research and clinical practice. During his master education additional courses in electrocardiography, radioprotection and dosimetrics and neurosurgery were attended. As a resident he successfully passed the test of English as a foreign language and the United States Medical Licensing Exams steps 1 and 2. After being recognized as a specialist by the Belgian Board of Anesthesiology in 2005 he successfully completed postgraduate training in intensive care medicine in 2007 and in emergency medicine in 2009. Then he followed training in pain therapy in the University Hospital of Maastricht (Prof. dr. M. van Kleef), in the Rijnstate Ziekenhuis in Arnhem (dr. J.W. Kallewaard and dr. M. Terheggen), at the Radboud University Nijmegen Medical Centre (dr. A. Wolf) and at the Spinal Diagnostics and Treatment Center in Daly City, CA in the United States (dr. R. Derby). In 2006 he passed the international examination of interventional pain management of the World Institute of Pain and became a Fellow of Interventional Pain Practice.

Since 2007 he is scientifically associated with the Donders Institute for Brain, Cognition and Behaviour, Center for Neuroscience, Radboud University Nijmegen and the department of Pain and Palliative Medicine at the Radboud University Nijmegen Medical Center where he started as a PhD fellow (supervisors: Prof. dr. K.C.P. Vissers, Prof. dr. E.W. Roubos, Prof. dr. T.L. Kozicz, dr. J Van Zundert).

He is frequently asked for lectures at national and international scientific meetings and is a reviewer for several peer-reviewed journals.

Pascal Vanelderen is married to Eva Vansummeren, psychiatrist, and has one son Viktor.



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